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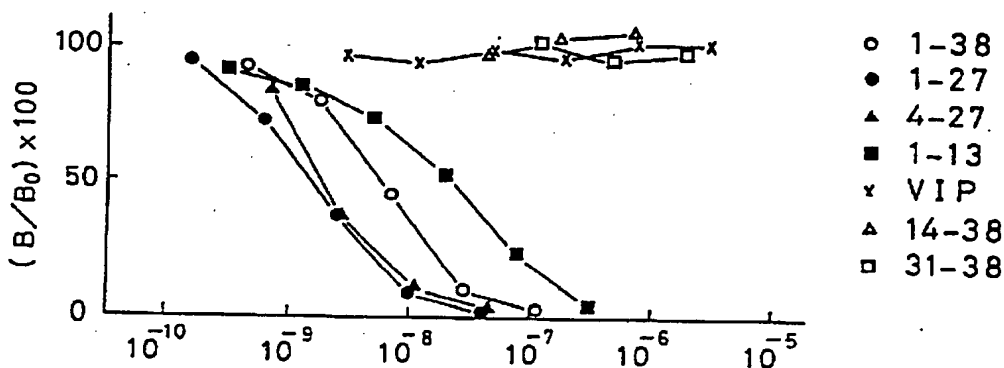


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(54) Title: ANTIBODY TO PITUITARY ADENYLATE CYCLASE ACTIVATING PEPTIDE-PACAP, HYBRIDOMA AND ASSAY FOR PACAP

(a) PA-1Na(class Ia)



(57) Abstract

Disclosed are a monoclonal antibody having affinity for PACAP, a partial peptide thereof, a precursor thereof or VIP; a hybridoma cell which produces the above monoclonal antibody; and an immunoassay for assaying PACAP by a competitive method or a sandwich method using the above antibody, whereby PACAP can be specifically detected with high sensitivity.

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Antibody to pituitary adenylate cyclase activating peptide-PACAP, hybridoma and assay for PACAP.

#### BACKGROUND OF THE INVENTION

The present invention relates to an antibody which is novel and useful in that it has specific affinity for PACAP, and more particularly to an antibody useful for development of assays of PACAP on the basis of antigen-antibody reactions or for diagnosis and treatment of diseases related to PACAP.

Various hormones secreted by brain hypothalami and hypophyses have been known. Examples thereof include thyrotropin releasing hormone, luteinizing hormone releasing hormone, somatostatin, adrenocorticotrophic hormone, growth hormone and prolactin. Action thereof has been studied in detail. Recently, a novel bioactive substance of hypothalamic origin other than these hormones was studied based upon adenylate cyclase activity, and consequently a peptide consisting of 38 amino acid residues which had not been reported till then was discovered from sheep hypothalami. This peptide was named "PACAP38NH<sub>2</sub>" and has a structure represented by the following formula:

His Ser Asp Gly Ile Phe Thr Asp Ser Tyr Ser Arg Tyr Arg  
Lys Gln Met Ala Val Lys Lys Tyr Leu Ala Ala Val Leu Gly Lys  
Arg Tyr Lys Gln Arg Val Lys Asn Lys-NH<sub>2</sub>

It was disclosed in applications for patents (Japanese Patent Application Nos. 1-155791/1990 and 1-284771/1990) on cDNA of sheep PACAP38, and an application for a patent (Japanese Patent Application No. 1-259924/1990) on the

- 2 -

partial structure of cDNA of human PACAP38 that the amino acid sequence of the mature portion of sheep PACAP38 was the same as that of human PACAP38, and that some amino acids of the precursors thereof were substituted. It is deduced from  
5 the position of continuous basic amino acids shown in the cDNA sequence of PACAP38NH<sub>2</sub> that PACAP27NH<sub>2</sub>, in addition to PACAP38NH<sub>2</sub>, will exist as a peptide cut out of the precursor.

In fact, according to subsequent studies PACAP27NH<sub>2</sub> was  
10 also isolated from sheep hypothalami, in addition to PACAP38NH<sub>2</sub>. The structure thereof is represented by the following formula:

PACAP27NH<sub>2</sub>

His Ser Asp Gly Ile Phe Thr Asp Ser Tyr Ser Arg Tyr Arg  
15 Lys Gln Met Ala Val Lys Lys Tyr Leu Ala Ala Val Leu-NH<sub>2</sub>

PACAP38NH<sub>2</sub> and PACAP27NH<sub>2</sub> are hereinafter represented by the general term of "PACAP". The 28 amino acid residues on the N-terminal side of PACAP38NH<sub>2</sub> containing PACAP27NH<sub>2</sub> show 68% homology with vasoactive intestinal polypeptide  
20 (VIP) well known as a brain-gut peptide. However, it has been reported that the adenylate cyclase activating activity of PACAP exceeds at least 1,000 times that of VIP.

Thus, the action of PACAP is anticipated to be different from that of VIP, and a deep interest is taken in  
25 the physiological role thereof and the relation thereof to the pathology.

Although the interest in PACAP is increased as

described above, basic physiological information such as existing sites other than hypothalami of PACAP and a plasma level thereof is scarcely obtained, and the relation thereof to the pathology is also unknown. This is mainly caused by  
5 that any monoclonal antibodies specifically recognizing PACAP have hitherto not been prepared and that any immunoassays for assaying PACAP specifically and highly sensitively have not been developed. These immunological procedures are considered to be one of the most effective  
10 means to study PACAP, particularly the metabolic pathways thereof, the secretory mechanism thereof, the receptor system thereof, the relation thereof to the pathology and the like collectively. The establishment of these procedures has therefore been earnestly desired in various  
15 fields.

Previously, competitive radioimmunoassays (RIA) generally using one kind of antibody and enzyme immunoassays (EIA) have been developed and employed to assay low molecular weight peptides such as PACAP. On the other hand,  
20 sandwich immunoassays using two kinds of antibodies have the advantages of (1) improving the specificity of assay systems because of the use of two kinds of antibodies and (2) being little affected by nonspecific interfering factors because of the use of the antibodies in large excess to substances  
25 to be assayed. Until now, however, it has been unknown at all whether or not the low molecular weight peptides having no disulfide linkage such as PACAP can be assayed with high

sensitivity by the sandwich methods. Namely, in case of the low molecular weight peptides having no disulfide linkage such as PACAP, the possibility is conceivable that binding sites of two kinds of antibodies are in so close proximity to each other as to exert influences such as steric hindrance, which results in difficulty of the establishment of highly sensitive sandwich methods.

#### SUMMARY OF THE INVENTION

The present inventors prepared polyclonal and monoclonal antibodies having affinity for PACAP and having different reaction specificity for partial peptides of PACAP and VIP, and developed an immunoassay which can specifically detect PACAP with high sensitivity using the antibodies and which can fractionate and determine PACAP38NH<sub>2</sub> and PACAP27NH<sub>2</sub>.

In accordance with the present invention, there are provided a monoclonal antibody having affinity for PACAP, a partial peptide of PACAP, a precursor of PACAP or VIP; a hybridoma cell which produces the above monoclonal antibody; and an immunoassay for assaying PACAP by a competitive method or a sandwich method using the above antibody.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing antibody titer to PACAP38NH<sub>2</sub> in mouse antisera;

Fig. 2 is a graph showing antibody titer to PACAP(11-27)NH<sub>2</sub> in rabbit antisera;

Fig. 3 is a graph showing the results assayed for

PACAP38 and related peptides by a competitive method-enzyme immunoassay using antibodies of the present invention, thereby clarifying recognition sites of the antibodies of the present invention;

5 Figs. 4 to 6 are graphs showing the results detected for PACAP38NH<sub>2</sub> by sandwich method-enzyme immunoassays using antibodies of the present invention;

Fig. 7 is a graph showing the reactivity of PACAP and related peptides in the sandwich method-enzyme immunoassay  
10 of the present invention;

Figs. 8 to 11 are graphs showing the reactivity of PACAP27NH<sub>2</sub> and related peptides in the sandwich method-enzyme immunoassays; and

Fig. 12 is a graph showing the results of examination  
15 of the neutralization activity of anti-PACAP antibodies to PACAP38NH<sub>2</sub> by the use of cultured cells.

Fig. 13 shows immunoblot analysis of the products of E. coli transfected with the expression plasmid containing human prepro-PACAP cDNA.

20 DESCRIPTION OF THE PREFERRED EMBODIMENTS

As the partial peptides of PACAP, any peptides may be used as long as they have partial sequences of PACAP. Examples of such peptides include peptides corresponding to an N-terminal portion of PACAP, such as PACAP(1-13) having  
25 the following sequence:

His Ser Asp Gly Ile Phe Thr Asp Ser Tyr Ser Arg Tyr,  
peptides corresponding to a region from the N-terminal

portion to a central portionb of PACAP, such as PACAP(4-27) having the following sequence:

Gly Ile Phe Thr Asp Ser Tyr Ser Arg Tyr Arg Lys Gln Met  
Ala Val Lys Lys Tyr Leu Ala Ala Val Leu,

- 5 peptides corresponding to a region from a C-terminal portion to a central portion of PACAP38NH<sub>2</sub>, such as PACAP(14-38) having the following sequence:

Arg Lys Gln Met Ala Val Lys Lys Tyr Leu Ala Ala Val Leu  
Gly Lys Arg Tyr Lys Gln Arg Val Lys Asn Lys,

- 10 peptides corresponding to the C-terminal portion of PACAP38NH<sub>2</sub>, such as PACAP(31-38) having the following sequence:

Tyr Lys Gln Arg Val Lys Asn Lys, and

- 15 peptides corresponding to a region from a central portion to a C-terminal portion of PACAP27, such as PACAP(11-27) having the following sequence:

Ser Arg Tyr Arg Lys Gln Met Ala Val Lys Lys Tyr Leu Ala  
Ala Val Leu-NH<sub>2</sub>

- 20 Of these peptides, it is preferred that the C-termini of PACAP(31-38) and PACAP(11-27) have amide forms, and the C-termini of the other peptides may have either amide forms or free carboxylic acid forms. The amide form is represented by giving NH<sub>2</sub> as PACAP(31-38)NH<sub>2</sub>, and the free carboxylic acid form is given no symbol or represented by  
25 giving OH, for example, PACAP(31-38)OH.

Examples of a precursor of PACAP include a precursor of human PACAP consisting of an amino acid sequence represented



- 7 -

by the following formula or a portion thereof:

Met Thr Met Cys Ser Gly Ala Arg Leu  
 Ala Leu Leu Val Tyr Gly Ile Ile Met His  
 Ser Ser Val Tyr Ser Ser Pro Ala Ala Ala  
 5 Gly Leu Arg Phe Pro Gly Ile Arg Pro Glu  
 Glu Glu Ala Tyr Gly Glu Asp Gly Asn Pro  
 Leu Pro Asp Phe Gly Gly Ser Glu Pro Pro  
 Gly Ala Gly Ser Pro Ala Ser Ala Pro Arg  
 Ala Ala Ala Ala Trp Tyr Arg Pro Ala Gly  
 10 Arg Arg Asp Val Ala His Gly Ile Leu Asn  
 Glu Ala Tyr Arg Lys Val Leu Asp Gln Leu  
 Ser Ala Gly Lys His Leu Gln Ser Leu Val  
 Ala Arg Gly Val Gly Gly Ser Leu Gly Gly  
 Gly Ala Gly Asp Asp Ala Glu Pro Leu Ser  
 15 Lys Arg His Ser Asp Gly Ile Phe Thr Asp  
 Ser Tyr Ser Arg Tyr Arg Lys Gln Met Ala  
 Val Lys Lys Tyr Leu Ala Ala Val Leu Gly  
 Lys Arg Tyr Lys Gln Arg Val Lys Asn Lys  
 Gly Arg Arg Ile Ala Tyr Leu

20 The present inventors made various investigations to  
 produce the monoclonal antibodies and the polyclonal  
 antibodies to PACAP. As a result, the antibodies roughly  
 classified into 5 classes were obtained.

The antibodies classified as class I recognize the N-  
 25 terminal portion of PACAP. Namely, they react with  
 PACAP38NH<sub>2</sub>, PACAP27NH<sub>2</sub>, PACAP(1-13) and PACAP(4-27), and do  
 not react with PACAP(14-38)NH<sub>2</sub> and PACAP(31-38)NH<sub>2</sub>.

- 8 -

The antibodies classified as class II recognize the region from the N-terminal portion to the central portion of PACAP. Namely, they react with PACAP38, PACAP27NH<sub>2</sub> and PACAP(4-27), and do not react with PACAP(1-13),

5 PACAP(14-28)NH<sub>2</sub> and PACAP(31-38)NH<sub>2</sub>.

The antibodies classified as class III recognize the region from the C-terminal portion to the central portion of PACAP. Namely, they react with PACAP38NH<sub>2</sub> and PACAP(14-38)NH<sub>2</sub>, and do not react with PACAP27NH<sub>2</sub>, PACAP(1-13),

10 PACAP(4-27) and PACAP(31-38)NH<sub>2</sub>.

The antibodies classified as class IV recognize the C-terminal portion of PACAP38NH<sub>2</sub>. Namely, they react with PACAP38NH<sub>2</sub>, PACAP(14-38)NH<sub>2</sub> and PACAP(31-38)NH<sub>2</sub>, and do not react with PACAP27NH<sub>2</sub>, PACAP(4-27) and PACAP(1-13).

15 The antibodies classified as class V recognize the C-terminal portion of PACAP27NH<sub>2</sub>. Namely, they are antibodies to PACAP(11-27)NH<sub>2</sub>.

The antibodies classified as class I are further classified into class Ia and class Ib. The antibodies

20 classified as class Ia exhibit only a cross reactivity with VIP of less than 0.5%, and the antibodies classified as class Ib exhibit a cross reactivity with VIP of 0.5% or more.

Many of the antibodies classified as class II show only

25 a cross reactivity with VIP of less than 0.5%, and many of the antibodies classified as classes III to V exhibit only a cross reactivity with VIP of less than 0.01%.

- 9 -

These antibodies can be used in ordinary tissue staining or competitive immunoassays. The present inventors further made various investigations to develop an excellent immunoassay, and consequently developed the sandwich  
5 immunoassay in which to kinds of these monoclonal or polyclonal antibodies are used in combination.

In the immunoassay for assaying PACAP by the sandwich method of the present invention, each of the antibodies used in a primary reaction (a reaction of an antibody for a solid  
10 phase with a substance to be tested) and a secondary reaction (a reaction of a labeled antibody with the substance to be tested) may be either the polyclonal antibody or the monoclonal antibody. It is however preferred that one of them is the antibody which recognizes  
15 the N-terminal portion of PACAP38NH<sub>2</sub> (class I), the region from the N-terminal portion to the central portion (class II), the region from the C-terminal portion to the central portion (class III) or C-terminal portion (class IV) and the other is the antibody which recognizes regions other than  
20 the regions described above.

Namely, the present inventors discovered that PACAP38NH<sub>2</sub> was detected with high sensitivity in combinations of class I and class III, class II and class IV, and class I and class IV, or in the sandwich method  
25 using the antibodies in combination which did not recognize the regions adjacent to each other. The present inventors further discovered that PACAP38NH<sub>2</sub> was also detected in

- 10 -

combinations of class I and class II, class II and class III, and class III and class IV, or in the sandwich method using the antibodies in combination which recognize the regions adjacent to each other, and particularly that

5 PACAP38NH<sub>2</sub> was detected with high sensitivity in the sandwich method in which the antibody of class II was used in combination with the antibody of class III. The sandwich immunoassay is specific for PACAP38NH<sub>2</sub>. For example, in the sandwich immunoassay using PA-6Na, one of the antibodies  
10 belonging to class II, and PA-2Ca, one of the antibodies belonging to class III, it was discovered that the cross reactivity with other peptides having homology with VIP and PACAP38NH<sub>2</sub> was 0.001% or less. Examples of such peptides include growth hormone releasing hormone (GRF) having the  
15 following sequence:

Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-Arg-Leu-NH<sub>2</sub>,

20 and secretin, a typical gastrointestinal hormone having the following sequence:

His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Glu-Gly-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH<sub>2</sub>  
(molecular weight: 3039.4)

25 In the immunoassay for assaying PACAP27NH<sub>2</sub> by the sandwich method of the present invention, each of the antibodies used in the primary and secondary reactions may

- 11 -

be either the polyclonal antibody or the monoclonal antibody. It is however preferred that one of them is the antibody which recognizes the N-terminal portion of PACAP27NH<sub>2</sub> (class I), the region from the N-terminal portion  
5 to the central portion (class II) or C-terminal portion (class V) and the other is the antibody which recognizes regions other than the regions described above. Also in this sandwich immunoassay of the present invention, PACAP27NH<sub>2</sub> can be detected without cross reaction with VIP,  
10 GRF or secretin (the cross reactivity is 0.001% or less). The sandwich immunoassay using either the antibody of class I or that of class II in combination with either the antibody of class III or that of class IV is specific for PACAP38NH<sub>2</sub> and does not cross react with PACAP27NH<sub>2</sub>. On the  
15 other hand, the immunoassay for assaying PACAP27NH<sub>2</sub> by the sandwich method using either the antibody of class I or that of class II in combination with the antibody of class V shows a cross reactivity with PACAP38NH<sub>2</sub> as low as 0.22 to 3.6% by weight ratio or 0.31 to 5% by molar ratio. By using  
20 these immunoassays, therefore, PACAP38NH<sub>2</sub> and PACAP27NH<sub>2</sub> can be fractionated and determined.

The polyclonal antibody used in the present invention is usually prepared by producing a complex comprising a carrier protein and PACAP or a partial peptide of PACAP,  
25 which acts as an immunogen. Next, animals are inoculated with this complex for immunization. The substance containing an anti-PACAP or anti-PACAP partial peptide

- 12 -

antibody from the immunized animals is recovered, and the antibody is then separated and purified.

The monoclonal antibody of the present invention is prepared by selecting individuals having high antibody titer from the above-mentioned immunized animals, recovering spleens or lymphatic corpuscles therefrom 2 to 5 days after the final immunization, fusing antibody producing cells contained therein with myeloma cells, and selecting hybridoma cells which stably produce an antibody having high titer to obtain monoclonal hybridoma cells.

Both of natural purified samples and synthetic samples can be used as immunogens. PACAP and portions thereof are used. Compounds containing the structure of PACAP or portions of PACAP are used as the immunogens in some cases.

The various peptides used in the present invention can be prepared by peptide synthesis methods known to those skilled in the art. Either the solid phase synthesis methods or the liquid phase synthesis methods may be used. Examples of the peptide synthesis methods include methods described in B. Merrifield [J. Am. Chem. Soc. 85, 2149 (1963)], M. Bodanszky and M. A. Ondetti [Peptide Synthesis, Interscience Publishers, New York (1966)], Schroder and Lubke [The Peptide, Academic Press, New York (1965)], N. Izumiya et al. [Fundamentals and Experiments of Peptide Synthesis, Maruzen (1985)] and H. Yajima and S. Sakakibara [Course of Biochemical Experiments 1, Chemistry of Proteins IV 205 (1977)].

- 13 -

For example, when PACAP38NH<sub>2</sub> or a partial peptide of PACAP38NH<sub>2</sub> is synthesized by the solid phase methods, using any of the insoluble resins known in the art such as chloromethyl resins, 4-methylbenzhydrylamine resins and  
5 4-oxymethylphenylacetamidomethyl resins, protected amino acids are successively condensed to the C-terminal side of PACAP38NH<sub>2</sub> or of the partial peptide of PACAP38NH<sub>2</sub> according to methods known in the art. Then, all protecting groups are removed by hydrogen fluoride treatment, followed by  
10 purification by methods known in the art such as high performance liquid chromatography, whereby the desired PACAP38NH<sub>2</sub> or partial peptide of PACAP38NH<sub>2</sub> can be obtained.

For example, the N-protected amino acids can be produced by protecting  $\alpha$ -amino groups with Boc groups, the  
15 hydroxyl groups of serine and threonine with Bzl groups, the  $\omega$ -carboxylic acid groups of glutamic acid and aspartic acid with OBzl groups, the  $\epsilon$ -amino group of lysine with a Cl-Z group, the hydroxyl group of tyrosine with a Br-Z group, the guanido group of arginine with a Tos group, and  
20 the imidazole group of histidine with a Tos group.

The abbreviations used in this specification, are the abbreviations adopted by IUPAC-IUB Commission on Biochemical Nomenclature or commonly used in the art are employed. For example, the following abbreviations are used. When an  
25 optical isomer is capable of existing with respect to the amino acids, the L-form is represented unless otherwise specified.

	PAM	: Phenylacetamidomethyl
	BHA	: Benzhydramine
	Boc	: t-Butyloxycarbonyl
	Cl-Z	: 2-Chloro-benzyloxycarbonyl
5	Br-Z	: 2-Bromo-benzyloxycarbonyl
	Bzl	: Benzyl
	OBzl	: Benzyl ester
	Tos	: p-Toluenesulfonyl
	HOBt	: 1-Benzotriazole
10	DCC	: N,N'-Dichlorohexylcarbodiimide
	Gly	: Glycine
	Ala	: Alanine
	Val	: Valine
	Leu	: Leucine
15	Ile	: Isoleucine
	Ser	: Serine
	Thr	: Threonine
	Cys	: Cysteine
	Met	: Methionine
20	Glu	: Glutamic acid
	Asp	: Aspartic acid
	Lys	: Lysine
	Arg	: Arginine
	His	: Histidine
25	Phe	: Phenylalanine
	Tyr	: Tyrosine
	Trp	: Tryptophan



- 15 -

Pro : Proline

Asn : Asparagine

Gln : Glutamine

With respect to the protein complexes comprising the  
5 immunogens and the carrier proteins used for immunization of  
mammals, any type of carrier proteins may be coupled with  
haptens in any ratio, as long as the antibodies can be  
produced effectively to the haptens coupled with the carrier  
proteins to be immunized. For example, bovine serum  
10 albumin, bovine thyroglobulin or hemocyanin is coupled with  
the hapten in a weight ratio of 0.1 to 20, and preferably 1  
to 5, per 1 of the hapten.

Various condensing agents may be used for the coupling  
of the haptens and the carrier proteins. In particular,  
15 glutaraldehyde, carbodiimide active esters, maleimide active  
esters and active ester reagents containing thiol groups or  
dithiopyridyl groups are advantageously used.

The condensed products are given alone or with carriers  
or diluents to warm-blooded animals at sites where the  
20 antibodies are capable of being produced. In giving the  
condensed products, Freund's complete adjuvant or Freund's  
incomplete adjuvant may be given to enhance the antibody  
productivity. The condensed products are usually given once  
every 2 to 6 weeks, 3 to 6 times in all.

25 The warm-blooded animals used therein include, for  
example, monkeys, rabbits, dogs, guinea pigs, mice, rats,  
sheep, goats and chickens.

The antibodies are harvested from the blood, the ascites and the like (preferably from the blood) of the warm-blooded animals immunized by the methods described above. The titer of the anti-PACAP antibody in the  
5 antiserum is determined, for example, by reacting labeled PACAP described below with the antiserum, and then measuring the activity of a labeling agent bound to the antibody. The antibodies are separated and purified according to methods for separating and purifying immunoglobulin which are known  
10 in the art. Such methods include salt precipitation, alcohol precipitation, isoelectric precipitation, electrophoresis, adsorption and desorption using an ion exchanger (for example, DEAE), ultracentrifugation, gel filtration and specific purifying methods for obtaining  
15 antibodies by recovering the antibodies alone by using active adsorbents such as antigen-antibody complexes, protein A and protein G and then breaking the binding.

The antibodies thus obtained comprise mainly of IgG and also contain additional immunoglobulins such as IgM and IgA.

20 The anti-PACAP antibody producing hybridoma cells of the present invention can be prepared by selecting individuals having high antibody titer from the warm-blooded animals such as mice immunized similarly to the above method for preparing the polyclonal antibody, recovering spleens or  
25 lymphatic corpuscles therefrom 2 to 5 days after the final immunization, and fusing antibody producing cells contained therein with myeloma cells. The cells may be fused

according to methods known in the art, for example, the method of Kohler and Milstein [Nature 256, 495 (1975)]. Fusion accelerators include polyethylene glycol (PEG) and Sendai virus. In particular, PEG is preferably used.

5       The myeloma cells include, for example, NS-1, P3U1 and SP2/0. In particular, P3U1 is preferably used. The ratio of the number of the antibody producing cells (splenic cells) to the myeloma cells is preferably about 1:1 to 20:1.

10       PEG(preferably PEG 1,000 to PEG 6,000) is added in a concentration of about 10 to 80%, and incubated at 20 to 40°C, preferably 30 to 37°C for 1 to 10 minutes, whereby the cell fusion can be effectively performed.

15       The anti-PACAP antibody producing hybridoma cells can be screened by various methods known in the art. Examples of such methods include an enzyme immunoassay (EIA) which comprises adding a hybridoma culture supernatant to a solid phase (for example, a microplate) allowed to adsorb PACAP or the partial peptide thereof, then adding an anti-immunoglobulin antibody (when mouse cells are used for the  
20 cell fusion, an anti-mouse immunoglobulin antibody is employed) labeled with horseradish peroxidase (HRP) or protein-A thereto, and detecting the anti-PACAP monoclonal antibody; and an enzyme immunoassay (EIA) which comprises adding a hybridoma culture supernatant to a solid phase  
25 allowed to adsorb an anti-immunoglobulin antibody or protein A, then adding HRP-labeled PACAP, and detecting the anti-PACAP monoclonal antibody bound to the solid phase.

- 18 -

Selection and breeding of the anti-PACAP monoclonal antibodies are usually achieved by addition of HAT (hypoxanthine, aminopterin and thymidine) and by use of a medium for animal cells containing 10 to 20% fetal calf serum, such as RPMI 1640. The antibody titer of the hybridoma culture supernatant can be assayed similarly to the above-mentioned method for assaying the titer of the anti-PACAP antibody in antiserum.

Isolation and purification of the anti-PACAP monoclonal antibodies are conducted in accordance with methods for isolating and purifying immunoglobulin, similar to the isolation and purification of the polyclonal antibodies described above.

The anti-PACAP polyclonal antibody reactive to a partial region of PACAP can be prepared by the above-mentioned method using a peptide corresponding to the partial region thereof as a hapten for immunization. Further, such anti-PACAP polyclonal antibody can also be prepared from the anti-PACAP polyclonal antibody prepared by using PACAP as a hapten, by use of affinity chromatography employing a column to which a peptide corresponding to the partial region thereof is bound.

Screening of hybridoma cells producing the anti-PACAP antibody reactive to a partial region of PACAP and hybridoma cells producing the anti-PACAP monoclonal antibody reactive to PACAP, but not reactive to the partial region thereof can be accomplished, for example, by assaying affinity of the

- 19 -

peptide corresponding to the above partial region for the antibody produced by the hybridoma cells.

By using the anti-PACAP monoclonal and polyclonal antibodies obtained above, assay and tissue staining of PACAP can be carried out. For these purposes, antibody molecules themselves may be used, and  $F(ab')_2$ , Fab' or Fab fractions of the antibody molecules may be used.

PACAP is usually assayed by competitive methods which will be described below. It is however preferable to use sandwich methods for the reason described above.

In the competitive methods, the anti-PACAP antibody obtained in the present invention is competitively reacted with a test solution and labeled PACAP, followed by measurement of the ratio of labeled PACAP bound to the antibody, thereby determining the amount of PACAP contained in the test solution.

The labeling agents for PACAP or for antibodies described below include radioisotopes, enzymes, fluorescent substances and luminous substances. The radioisotopes include, for example,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^3\text{H}$  and  $^{14}\text{C}$ . The enzymes which are stable and high in specific activity are preferably used. Examples of such enzymes include  $\beta$ -galactosidase,  $\beta$ -glucosidase, alkaline phosphatase, peroxidase and malate dehydrogenase. The fluorescent substances include fluorescamine and fluorescein isothiocyanate. The luminous substances include luminol, luminol derivatives, luciferin and lucigenin. Further, a

- 20 -

biotin-avidin system may also be used in order to bind the labeling agent to the antibody or PACAP.

When the activity of the above-mentioned labeling agents is assayed, it is necessary to separate labeled PACAP bound to the antibody from free labeled PACAP. This separation is hereinafter referred to as B/F separation for brevity. When the enzymes are used as the labeling agents, active adsorbents such as insolubilized antibodies to the anti-PACAP antibody or insolubilized protein A are advantageously used as reagents for the B/F separation. For example, an anti-IgG antibody (corresponding to the antibody to the anti-PACAP antibody) is used as the solid phase, and labeled PACAP binds to the anti-IgG antibody of the solid phase through the above-mentioned antibody reactive thereto to measure the labeling agent on the solid phase. When the enzymes are used as the labeling agents, the activity of the enzymes on an insolubilized carrier is assayed by ordinary colorimetric methods or fluorescent methods. When the radioisotopes and the like are used as the labeling agents, reagents such as antibodies to the anti-PACAP antibody which are not insolubilized, sodium sulfate, dextran charcoal powder and polyethylene glycol are used for the B/F separation, in addition to the above-mentioned reagents. In any methods, the activity of the labeling agent in the supernatant or in the precipitate is assayed.

The above-mentioned insolubilization may be achieved by physical adsorption or chemical bonding usually used to

- 21 -

insolubilize or immobilize polysaccharides such as agarose, dextran and cellulose; synthetic resins such as polystyrene, polyacrylamide and silicone; and glass.

In the competitive methods, the anti-PACAP antibody,  
5 the test solution, labeled PACAP and the reagent for B/F separation can be reacted in any order. Also, all or a part of them may be reacted at the same time. It is however preferable that at least labeled PACAP is added to the reaction system simultaneously with the reaction of the test  
10 solution and the anti-PACAP antibody, or after the reaction.

The reagents for the B/F separation such as sodium sulfate, dextran charcoal powder and polyethylene glycol are mainly added to the reaction system at the final stage thereof.

On the other hand, in the sandwich methods, the test  
15 solution is brought into contact with (or reacted with) the insolubilized anti-PACAP antibody (the primary reaction), and further the labeled anti-PACAP antibody is reacted therewith (the secondary reaction), followed by assay of the activity of the labeling agent on the insolubilized carrier,  
20 whereby the amount of PACAP in the test solution can be determined. The primary and secondary reactions may be conducted at the same time or at different time. The labeling agents and the insolubilizing methods can conform to those described above.

25 As the anti-PACAP antibody used in the secondary reaction, it is preferred to use the antibody different from the anti-PACAP antibody used in the primary reaction in the

- 22 -

site to which PACAP binds.

Namely, the antibodies used in the primary and secondary reactions may be polyclonal or monoclonal antibodies, respectively. For example, however, when the  
5 antibody used in the primary reaction recognizes the C-terminal portion of PACAP38NH<sub>2</sub> (class IV), it is preferred to use in the secondary reaction the antibody which recognizes portions or regions other than the C-terminal portion, namely recognizes the N-terminal portion (class I),  
10 the region from the N-terminal portion to the central portion (class II) or the region from C-terminal portion to the central portion (class III).

In the sandwich immunoassays, both of the antibody for solid phase and the antibody for labeling may be antibodies  
15 of any class and subclass, and may be F(ab')<sub>2</sub>, Fab' or Fab fractions which are obtained by removing Fc' or Fc fractions therefrom, as long as they have antibody activity.

In the sandwich immunoassays, when the monoclonal antibody is used, it is not always necessary to use one kind  
20 of antibody as the antibody for solid phase or for labeling.

For the purpose of improving assaying sensitivity, mixtures of two or more kinds of antibodies can be used.

Further, the immunoassays using the antibodies which are obtained according to the present invention can be used  
25 for diagnosis and treatment of diseases related to PACAP.

Humors such as plasma, serum, urine, cerebrospinal fluid, ascites, pleural fluid and amniotic fluid, sputum and



- 23 -

feces can be used as test samples. These samples can be used for the immunoassays as such or with concentration after dilution or extraction with various buffers.

Any buffers or organic solvents can be used as solvent  
5 for dilution or extraction of the samples. Preferred examples thereof include buffers for immunoassay, water, physiological saline, acetate buffer, acetone, chloroform-methanol and these solutions containing surface active agents. After extraction, the samples are sometimes heat  
10 treated. The samples may be concentrated directly under reduced pressure or under ordinary pressure in a stream of nitrogen. Also, the samples may be added to carriers for ion exchange or for reverse-phase chromatography, or to anti-PACAP antibody-bound carriers, and then eluted under  
15 appropriate conditions, followed by concentration under reduced pressure or under ordinary pressure in a stream of nitrogen. The carriers for reverse-phase chromatography include C2, C8, C18 and phenyl cartridges. It is particularly preferable to use the anti-PACAP antibody-  
20 bound carriers as the carriers for concentration. Condensates are dissolved in the buffers for immunoassay, and then subjected to the immunoassays.

Further, the anti-PACAP antibodies obtained in the present invention can also be used for immunohistochemical  
25 station of PACAP. Methods thereof can be conducted, for example, in accordance with direct methods using the labeled anti-PACAP antibodies, and indirect methods using the anti-

- 24 -

PACAP antibodies and the labeled antibodies to the anti-PACAP antibodies.

Furthermore, of the anti-PACAP antibodies obtained in the present invention, the antibody which can neutralize the  
5 adenylate cyclase activity of PACAP can be used as a specific neutralizing antibody.

As methods for screening an antibody specifically depressing the activity of PACAP from the anti-PACAP antibodies, any methods for detecting the pharmacological  
10 activity of PACAP can be used. Examples of such methods include an in vitro assay system in which measurements are made based on the adenylate cyclase activity of PACAP in primary culture of hypophyses or in culture systems of various cells containing brown cytoma cell strain PC12h, and  
15 an in vivo assay system in which measurements are made based on the vasodepressor activity of PACAP to the experimental animals.

The antibodies specifically depressing the activity of PACAP may be antibodies of any class, such as IgG, IgA and  
20 IgM, and may be Fab' of Fab fractions which are obtained by removing Fc' or FC regions therefrom, or polymers of the fractions. A chimera antibody can also be used which is obtained by fusing a variable gene region of a monoclonal antibody being specifically capable of depressing the  
25 activity of PACAP with a constant gene region of human immunoglobulin, followed by expression as a recombinant.

Hybridoma cells obtained in Example 8 described below

were deposited with the Institute for Fermentation, Osaka, Japan (IFO) on February 27, 1990, and with the Fermentation Research Institute, the Agency of Industrial Science and Technology, the Ministry of International Trade and Industry, Japan (FRI) on March 16, 1991, under the following accession numbers.

	Hybridoma cell	IFO	FERM-BP (FRI)
	PA-1N	50225	2811
	PA-3N	50226	2812
10	PA-5N	50227	2813
	PA-6N	50228	2814
	PA-2C	50229	2815
	PA-1C	50230	2816

In the following Examples, antibodies obtained from the respective hybridoma cells are represented by giving a symbol "a" after the names of the cells.

#### Reference Example 1

##### Synthesis of PACAP38 NH<sub>2</sub>

PACAP38 NH<sub>2</sub> was synthesized by using 1.04 g (0.5 mmole) of a commercially available p-methyl BHA resin (Applied Biosystems Inc.) and a peptide synthesizer (Model 430A, Applied Biosystems Inc.).

A starting amino acid, Boc-Lys(Cl-Z), was activated with HOBt/DCC and then condensed to the resin. Thereafter, the Boc group on the resin was treated with 50% trifluoroacetic acid/methylene chloride to deprotect the amino group. To this free amino group, the following

- 26 -

protected amino acids activated with HOBt/DCC were condensed in turn according to the amino acid sequence of PACAP38:

Boc-Asn, Boc-Lys(Cl-Z), Boc-Val, Boc-Arg(Tos), Boc-Gln,  
Boc-Tyr(Br-Z), Boc-Gly, Boc-Leu, Boc-Ala, Boc-Met, Boc-  
5 Ser(Bzl), Boc-Asp(OBzl), Boc-Thr(Bzl), Boc-Phe, Boc-  
Ile, and Boc-His(Tos)

After the completion of each reaction, the residual amino groups were acetylated with acetic anhydride to obtain 2.42 g of a protected PACAP38 NH<sub>2</sub> resin.

10 0.51 g of the resulting protected PACAP38 NH<sub>2</sub> resin was treated with 5 ml of hydrogen fluoride in the presence of 0.6 g of p-cresol at 0°C for 60 minutes, followed by removal of excess hydrogen fluoride by distillation under reduced pressure. The residue was washed twice with 5 ml of ethyl  
15 ether, and then extracted with 6 ml of 50% aqueous acetic acid. The insoluble material was removed by filtration and washed with 5 ml of 50% aqueous acetic acid. The filtrate and the washings were combined, and the combined solution was concentrated to 2 to 3 ml. The concentrated solution  
20 was applied on a Sephadex LH-20 column (2 X 90 cm) for elution with 50% acetic acid. The main fractions were collected, followed by removal by distillation under reduced pressure. Then, the residue was dissolved in 100 ml of 0.1% aqueous trifluoroacetic acid. The resulting solution was  
25 subjected to a YMC-ODS AM120 S-50 resin column (1.6 X 7 cm) and eluted by a linear gradient of 0.1% aqueous trifluoroacetic acid and 50% acetonitrile containing 0.1%

- 27 -

trifluoroacetic acid.

The main fractions were combined, followed by lyophilization. Thus, 60 mg of white powder was obtained.

This powder was dissolved in 20 ml of 0.05 M aqueous

5 ammonium acetate. The resulting solution was subjected to a CM-Cellulofine resin column (1 X 6 cm) and eluted by a

linear gradient of from 0.05 M to 1 M ammonium acetate. The main fractions were combined. The combined solution was

subjected to a YMC-ODS column (2.6 X 7 cm) again and eluted

10 by a linear gradient of from 0% to 40% aqueous acetonitrile containing 0.1% trifluoroacetic acid. The fractions of 28% to 30% acetonitrile were collected, followed by

lyophilization. Thus, 21.6 mg of white powder was obtained.

Anal. for amino acids:

15 Asp 2.90(3), Thr 0.84(1), Ser 2.10(3), Glu 2.21(2),  
Gly 2.00(2), Ala 3.29(3), Val 3.19(3), Met 1.01(1),  
Ile 0.87(1), Leu 2.19(2), Tyr 3.93(4), Phe 0.92(1),  
Lys 7.18(7), His 0.96(1), Arg 4.19(4)

(M + H)<sup>+</sup> by mass spectrography (SIMS) : 4530

20 HPLC elution time : 19.6 minutes

Column conditions

Column: YMC-ODS (AM-301, S-5 120A)

Eluent: A (0.1% aqueous trifluoroacetic acid)

B (acetonitrile containing 0.1% trifluoroacetic  
25 acid)

A linear gradient elution from the eluent A to the  
eluent B for 50 minutes

Flow rate: 1.0 ml/minute

Reference Example 2Synthesis of PACAP27 NH<sub>2</sub>

PACAP27 NH<sub>2</sub> was synthesized by using 1.04 g (0.5 mmole) of a commercially available p-methyl BHA resin (Applied Biosystems Inc.) and a peptide synthesizer (Model 430A, Applied Biosystems Inc.).

A starting amino acid, Boc-Leu, was activated with HOBt/DCC and then condensed to the resin. Thereafter, the Boc group on the resin was treated with 50% trifluoroacetic acid/methylene chloride to deprotect the amino group. To this free amino group, the following protected amino acids activated with HOBt/DCC were condensed in turn according to the amino acid sequence of PACAP38 (1-27):

Boc-Val, Boc-Ala, Boc-Leu, Boc-Tyr(Br-Z), Boc-Lys(Cl-Z), Boc-Met, Boc-Gln, Boc-Arg(Tos), Boc-Ser(Bzl), Boc-Asp(OBzl), Boc-Thr(Bzl), Boc-Phe, Boc-Ile, and Boc-His(Tos)

After the completion of each reaction, the residual amino groups were acetylated with acetic anhydride to obtain 2.31 g of a protected PACAP27 NH<sub>2</sub> resin.

0.79 g of the resulting protected PACAP27 NH<sub>2</sub> resin was treated with 10 ml of absolute hydrogen fluoride in the presence of 1.2 g of p-cresol at 0°C for 60 minutes, followed by removal of excess hydrogen fluoride by distillation under reduced pressure. The residue was washed twice with 5 ml of ethyl ether, and then extracted with 5 ml of 50% aqueous acetic acid. The insoluble material was

- 29 -

removed by filtration and washed with 5 ml of 50% aqueous acetic acid. The filtrate and the washings were combined, and the combined solution was concentrated to 2 to 3 ml. The concentrated solution was applied on a Sephadex LH-20 column (2 X 75 cm) for elution with 50% acetic acid. The main fractions were collected, followed by distillation under reduced pressure. The residue was dissolved in 100 ml of 0.1% aqueous trifluoroacetic acid. The resulting solution was subjected to a YMC-ODS AM120 S-50 resin column (2.6 X 7 cm) and eluted by a liner gradient of 0.1% aqueous trifluoroacetic acid and 50% acetonitrile containing 0.1% trifluoroacetic acid. The main fractions were combined and the combined solution was applied onto a YMC-ODS column (2.6 X 7 cm) again and eluted by a linear concentration gradient with from 15 to 35% aqueous acetonitrile solution containing 0.1% trifluoroactetic acid. The acetonitrile 30 to 32% fractions were collected, followed by lyophilization. The resulting product was dissolved in 20 ml of 0.05M-aqueous ammonium acetate. The solution was applied onto a CM-Cellulofine resin column (1 X 6 cm) and eluted by a linear concentration gradient with water to 0.33 M - aqueous ammonium acetate.

The main fractions (0.18 to 0.22 M) were collected, followed by lyophilization. Thus, 20 mg of white powder was obtained.

Anal. for amino acids:

Asp 1.96(2), Thr 0.94(1), Ser 2.57(3), Glu 1.07(1),

- 30 -

Gly 0.95(1), Ala 3.00(3), Val 1.96(2), Met 0.88(1),  
Ile 0.88(1), Leu 1.93(2), Tyr 2.87(3), Phe 0.90(1),  
Lys 2.91(3), His 0.94(1), Arg 2.17(2)

(M + H)<sup>+</sup> by mass spectrography (SIMS): 3146.7

5 HPLS elution time: 21.2 minutes

Column conditions

Column: YMC-ODS (AM-301, S-5 120A)

Eluent: A (0.1% aqueous trifluoroacetic acid)

10 B (acetonitrile containing 0.1% trifluoroacetic  
acid)

A linear gradient elution from the eluent A to the  
eluent B for 50 minutes

Flow rate: 1.0 ml/minute

### Reference Example 3

15 Synthesis of PACAP27 OH (His ser Asp Gly Ile Phe Thr Asp  
Ser Tyr Ser Arg Tyr Arg Lys Gln Met Ala Val Lys Lys Tyr Leu  
Ala Ala Val Leu-OH)

PACAP27 OH was synthesized by using 0.60 g (0.5 mmole)  
of a commercially available Boc-Leu-OCH<sub>2</sub>-PAM resin (Applied  
20 Biosystems Inc.) and a peptide synthesizer (Model 430A,  
Applied Biosystems Inc.).

The Boc group on the resin was treated with 50%  
trifluoroacetic acid/methylene chloride to deprotect the  
amino group. To this free amino group, the following  
25 protected amino acids activated with HOBt/DCC were condensed  
in turn according to the amino acid sequence of PACAP27:

Boc-Val, Boc-Lys(Cl-Z), Boc-Arg(Tos), Boc-Gln,



- 31 -

Boc-Tyr(Br-Z), Boc-Gly, Boc-Leu, Boc-Ala, Boc-Met,  
Boc-Ser(Bzl), Boc-Asp(OBzl), Boc-Thr(Bzl), Boc-Phe,  
Boc-Ile, and Boc-His(Tos)

After the additional condensation by the amino acids  
5 activated by DCC or HOBt/DCC, the unreacted amino groups  
were acetylated with acetic anhydride to obtain 1.25 g of a  
protected PACAP27 OH resin.

0.65 g of the resulting protected PACAP27-OCH<sub>2</sub>-PAM  
resin was treated with 6 ml of absolute hydrogen fluoride in  
10 the presence of 1.0 g of p-cresol at 0°C for 60 minutes,  
followed by removal of excess hydrogen fluoride by  
distillation under reduced pressure. The residue was washed  
twice with 5 ml of ethyl ether, and then extracted with 5 ml  
of 50% aqueous acetic acid. The insoluble material was  
15 removed by filtration and washed with 5 ml of 50% aqueous  
acetic acid. The filtrate and the washings were combined,  
and the combined solution was concentrated to 2 to 3 ml  
under reduced pressure. The concentrated solution was  
applied on a Sephadex LH-20 column (2 X 75 cm) for elution  
20 with 50% acetic acid. The main fractions were collected,  
followed by distillation under reduced pressure. The  
residue was dissolved in 100 ml of 0.1% aqueous  
trifluoroacetic acid. The resulting solution was subjected  
to a YMC-ODS AM120 S-50 resin column (2.6 X 7 cm) and eluted  
25 by a liner gradient of 0.1% aqueous trifluoroacetic acid and  
50% acetonitrile containing 0.1% trifluoroacetic acid. The  
main fractions were combined and the combined solution was

- 32 -

applied onto a YMC-ODS column (2.6 X 7 cm) again and eluted by a linear concentration gradient with from 15 to 40% aqueous acetonitrile solution containing 0.1% trifluoroacetic acid. The acetonitrile 25 to 28% fractions were collected, followed by lyophilization. The resulting product was dissolved in 20 ml of 0.05M-aqueous ammonium acetate. The solution was applied onto a CM- Cellulofine resin column (1 X 6 cm) and eluted by a linear concentration gradient with 0.05M to 0.33M - aqueous ammonium acetate.

10 The main fractions were collected, followed by lyophilization. Thus, 20 mg of white powder was obtained.

Anal. for amino acids:

Asp 2.03(2), Thr 0.96(1), Ser 2.66(3), Glu 1.08(1),  
Gly 1.01(1), Ala 3.05(3), Val 1.98(2), Met 0.94(1),  
15 Ile 0.94(1), Leu 2.00(2), Tyr 2.96(3), Phe 0.95(1),  
Lys 2.99(3), His 1.03(1), Arg 2.25(2)

(M + H)<sup>+</sup> by mass spectrography (SIMS): 3147.9

HPLS elution time: 18.69 minutes

Column conditions

20 Column: YMC-ODS (AM-301, S-5 120A) (4.6 x 100)

Eluent: A (0.1% aqueous trifluoroacetic acid)

B (acetonitrile containing 0.1% trifluoroacetic acid)

A linear gradient elution from the eluent A to the  
25 eluent B for 25 minutes

Flow rate: 1.0 ml/minute

Example 1-1Synthesis of PACAP(14-38) NH<sub>2</sub>

PACAP(14-38) NH<sub>2</sub> was synthesized by using 1.04 g (0.5 mmole) of a commercially available p-methyl BHA resin (Applied Biosystems Inc.) and a peptide synthesizer (Model 430A, Applied Biosystems Inc.).

A starting amino acid, Lys(Cl-Z), was activated with HOBt/DCC and then condensed to the resin. Thereafter, the Boc group on the resin was treated with 50% trifluoroacetic acid/methylene chloride to deprotect the amino group. To this free amino group, the following protected amino acids activated with HOBt/DCC were condensed in turn according to the amino acid sequence of PACAP(14-38) NH<sub>2</sub>:

Boc-Asn, Boc-Lys(Cl-Z), Boc-Val, Boc-Arg(Tos), Boc-Gln, Boc-Tyr(Br-Z), Boc-Gly, Boc-Leu, Boc-Ala, Boc-Met

After the additional condensation by the amino acids activated by DCC or HOBt/DCC, the unreacted amino groups were acetylated with acetic anhydride to obtain 2.00 g of a protected PACAP(14-38) NH<sub>2</sub> resin.

0.48 g of the resulting protected PACAP(14-38) NH<sub>2</sub> resin was treated with 5 ml of absolute hydrogen fluoride in the presence of 0.48 g of p-cresol at 0°C for 60 minutes, followed by removal of excess hydrogen fluoride by distillation under reduced pressure. The residue was washed twice with 5 ml of ethyl ether, and then extracted with 5 ml of 50% aqueous acetic acid. The insoluble material was removed by filtration and washed with 5 ml of 50% aqueous

- 34 -

acetic acid. The filtrate and the washings were combined, and the combined solution was concentrated to 2 to 3 ml. The concentrated solution was applied on a Sephadex LH-20 column (2 X 75 cm) for elution with 50% acetic acid. The  
5 main fractions were collected, followed by distillation under reduced pressure. The residue was dissolved in 100 ml of 0.1% aqueous trifluoroacetic acid. The resulting solution was subjected to a YMC-ODS AM120 S-50 resin column (2.6 X 7 cm) and eluted by a liner gradient of 0.1% aqueous  
10 trifluoroacetic acid and 30% acetonitrile containing 0.1% trifluoroacetic acid. The main fractions were collected, followed by lyophilization. Thus, 20.2 mg of white powder was obtained.

Anal. for amino acids:

15 Asp 1.01(1), Glu 2.01(2), Gly 1.00(1), Ala 3.01(3),  
Val 2.85(3), Met 0.86(1), Leu 2.08(2), Tyr 1.98(2),  
Lys 6.37(7), Arg 3.24(3)

(M + H)<sup>+</sup> by mass spectrography (SIMS): 3003.6

HPLS elution time: 13.1 minutes

20 Column conditions

Column: YMC-ODS (AM-301, S-5 120A)

Eluent: A (0.1% aqueous trifluoroacetic acid)

B (acetonitrile containing 0.1% trifluoroacetic  
acid)

25 A linear gradient elution from the eluent A to the  
eluent B for 25 minutes

Flow rate: 1.0 ml/minute

- 35 -

Example 1-2

## Synthesis of PACAP(1-13) OH

PACAP(1-13) OH was synthesized by using 0.87 g (0.5 mmole) of a commercially available Boc-Tyr(Br-Z)-OCH<sub>2</sub>-PAM resin (Applied Biosystems Inc.) and a peptide synthesizer (Model 430A, Applied Biosystems Inc.).

The Boc group on the resin was treated with 50% trifluoroacetic acid/methylene chloride to deprotect the amino group. To this free amino group, the following protected amino acids activated with HOBt/DCC were condensed in turn according to the amino acid sequence of PACAP(1-13):

Boc-Arg(Tos), Boc-Tyr(Br-Z), Boc-Gly, Boc-Ser(Bzl), Boc-Asp(OBzl), Boc-Thr(Bzl), Boc-Phe, Boc-Ile, and Boc-His(Tos)

After the additional condensation by the amino acids activated by DCC or HOBt/DCC, the unreacted amino groups were acetylated with acetic anhydride to obtain 1.86 g of a protected PACAP(1-13)OCH<sub>2</sub>-PAM resin.

0.70 g of the resulting protected resin was treated with 10 ml of absolute hydrogen fluoride in the presence of 0.81 g of p-cresol at 0°C for 60 minutes, followed by removal of excess hydrogen fluoride by distillation under reduced pressure. The residue was washed twice with 5 ml of ethyl ether, and then extracted with 5 ml of 50% aqueous acetic acid. The insoluble material was removed by filtration and washed with 5 ml of 50% aqueous acetic acid. The filtrate and the washings were combined, and the

- 36 -

combined solution was concentrated to 2 to 3 ml under reduced pressure. The concentrated solution was applied on a Sephadex LH-20 column (2 X 75 cm) for elution with 50% acetic acid. The main fractions were collected, followed by  
5 distillation under reduced pressure. The residue was dissolved in 100 ml of 0.1% aqueous trifluoroacetic acid. The resulting solution was subjected to a YMC-ODS AM120 S-50 resin column (2.6 X 7 cm) and eluted by a liner gradient of 0.1% aqueous trifluoroacetic acid and 33% acetonitrile  
10 containing 0.1% trifluoroacetic acid. The main fractions were combined and the combined solution was purified again under the same column conditions. The main fractions were collected, followed by lyophilization. Thus, 38 mg of white powder was obtained.

15 Anal. for amino acids:

Asp 2.00(2), Thr 0.93(1), Ser 2.43(3), Glu 1.05(1),  
Gly 1.00(1), Tyr 1.82(2), Phe 1.02(1), His 1.31(1),  
Arg 1.12(1)

(M + H)<sup>+</sup> by mass spectrography (SIMS): 1547.5

20 HPLS elution time: 12.3 minutes

Column conditions

Column: YMC-ODS (AM-301, S-5 120A)

Eluent: A (0.1% aqueous trifluoroacetic acid)

B (acetonitrile containing 0.1% trifluoroacetic  
25 acid)

A linear gradient elution from the eluent A to the eluent B for 25 minutes

Flow rate: 1.0 ml/minute

Example 1-3

## Synthesis of PACAP(4-27) OH

PACAP(4-27) OH was synthesized by using 0.60 g (0.5 mmole) of a commercially available Boc-Leu-OCH<sub>2</sub>-PAM resin (Applied Biosystems Inc.) and a peptide synthesizer (Model 430A, Applied Biosystems Inc.).

The Boc group on the resin was treated with 50% trifluoroacetic acid/methylene chloride to deprotect the amino group. To this free amino group, the following protected amino acids activated with HOBt/DCC were condensed in turn according to the amino acid sequence of PACAP(4-27):

Boc-Lys(Cl-Z), Boc-Val, Boc-Arg(Tos), Boc-Gln,  
Boc-Tyr(Br-Z), Boc-Gly, Boc-Leu, Boc-Ala, Boc-Met,  
Boc-Ser(Bzl), Boc-Asp(OBzl), Boc-Thr(Bzl), Boc-Phe,  
Boc-Ile

After the additional condensation by the amino acids activated by DCC or HOBt/DCC, the unreacted amino groups were acetylated with acetic anhydride to obtain 1.08 g of a protected PACAP(4-27)OCH<sub>2</sub>-PAM resin.

0.29 g of the resulting protected resin was treated with 5 ml of absolute hydrogen fluoride in the presence of 0.49 g of p-cresol at 0°C for 60 minutes, followed by removal of excess hydrogen fluoride by distillation under reduced pressure. The residue was washed twice with 5 ml of ethyl ether, and then extracted with 5 ml of 50% aqueous acetic acid. The insoluble material was removed by filtration and washed with 5 ml of 50% aqueous acetic acid.

- 38 -

The filtrate and the washings were combined, and the combined solution was concentrated to 2 to 3 ml under reduced pressure. The concentrated solution was applied on a Sephadex LH-20 column (2 X 75 cm) for elution with 50% acetic acid. The main fractions were collected, followed by distillation under reduced pressure. The residue was dissolved in 100 ml of 0.1% aqueous trifluoroacetic acid. The resulting solution was subjected to a YMC-ODS AML20 S-50 resin column (2.6 X 7 cm) and eluted by a liner gradient of 15% acetonitrile containing 0.1% trifluoroacetic acid and 50% acetonitrile containing 0.1% trifluoroacetic acid. The main fractions were collected, followed by lyophilization to obtain 33 mg of white powder. The powder was dissolved in 20 ml of 0.05M-aqueous ammonium acetate. The solution was applied onto a CM-Cellulofine resin column (1 x 6 cm) and eluted by a linear gradient with water to 0.30M-aqueous ammonium acetate. The main fractions (0.18 to 0.22 M) were collected, followed by lyophilization. Thus, 33 mg of white powder was obtained.

Anal. for amino acids:

Asp 1.02(1), Thr 0.98(1), Ser 1.78(3), Glu 1.07(1),  
Gly 1.02(1), Ala 3.04(3), Val 1.89(2), Met 0.81(1),  
Ile 0.89(1), Leu 2.00(2), Tyr 2.91(3), Phe 0.90(1),  
Lys 2.89(3), Arg 2.20(2)

(M + H)<sup>+</sup> by mass spectrography (SIMS): 2808.5

HPLS elution time: 14.5 minutes



- 39 -

## Column conditions

Column: YMC-ODS (AM-301, S-5 120A)

Eluent: A (0.1% aqueous trifluoroacetic acid)

B (acetonitrile containing 0.1% trifluoroacetic acid)

5

A linear gradient elution from the eluent A to the eluent B for 35 minutes

Flow rate: 1.0 ml/minute

Example 1-4

10

Synthesis of PACAP(31-38) NH<sub>2</sub>

PACAP(31-38) NH<sub>2</sub> was synthesized by using 0.98 g (0.5 mmole) of a commercially available p-methyl BHA resin (Applied Biosystems Inc.) and a peptide synthesizer (Model 430A, Applied Biosystems Inc.).

15

A starting amino acid, Boc-Lys(Cl-Z), was activated with HOBt/DCC and then condensed to the resin. Thereafter, the Boc group on the resin was treated with 50% trifluoroacetic acid/methylene chloride to deprotect the amino group. To this free amino group, the following protected amino acids activated with HOBt/DCC were condensed in turn according to the amino acid sequence of PACAP(14-38) NH<sub>2</sub>:

20

Boc-Asn, Boc-Lys(Cl-Z), Boc-Val, Boc-Arg(Tos), Boc-Gln, Boc-Tyr(Br-Z)

25

After the additional condensation by the amino acids activated by DCC or HOBt/DCC, the unreacted amino groups were acetylated with acetic anhydride to obtain 2.00 g of a

- 40 -

protected PACAP(31-38)  $\text{NH}_2$  resin.

0.43 g of the resulting protected PACAP(31-38)  $\text{NH}_2$  resin was treated with 5 ml of absolute hydrogen fluoride in the presence of 0.6 g of p-cresol at  $0^\circ\text{C}$  for 60 minutes, followed by removal of excess hydrogen fluoride by distillation under reduced pressure. The residue was washed twice with 5 ml of ethyl ether, and then extracted with 5 ml of 50% aqueous acetic acid. The insoluble material was removed by filtration and washed with 5 ml of 50% aqueous acetic acid. The filtrate and the washings were combined, and the combined solution was concentrated to 2 to 3 ml. The concentrated solution was applied on a Sephadex LH-20 column (2 X 75 cm) for elution with 50% acetic acid. The main fractions were collected, followed by distillation under reduced pressure. The residue was dissolved in 100 ml of 0.1% aqueous trifluoroacetic acid. The resulting solution was subjected to a YMC-ODS AM120 S-50 resin column (2.6 X 7 cm) and eluted by a liner gradient of 0.1% aqueous trifluoroacetic acid and 33% acetonitrile containing 0.1% trifluoroacetic acid. The main fractions were collected, followed by lyophilization. Thus, 45 mg of white powder was obtained.

Anal. for amino acids:

Asp 1.02(1), Glu 1.05(1), Val 1.00(1), Tyr 0.90(1),  
Lys 2.98(3), Arg 1.12(1)

(M + H)<sup>+</sup> by mass spectrography (SIMS): 1062.7

HPLS elution time: 11.6 minutes

- 41 -

## Column conditions

Column: YMC-ODS (AM-301, S-5 120A)

Eluent: A (0.1% aqueous trifluoroacetic acid)

B (acetonitrile containing 0.1% trifluoroacetic  
acid)A linear gradient elution from the eluent A to the  
eluent mixture [A:B(4:1)] for 20 minutes

Flow rate: 1.0 ml/minute

Example 1-510 Synthesis of [Cys<sup>10</sup>]PACAP(11-27) NH<sub>2</sub>

[Cys<sup>10</sup>]PACAP(11-27) NH<sub>2</sub> was synthesized by using 0.66 g  
(0.5 mmole) of a commercially available p-methyl BHA resin  
(Applied Biosystems Inc.) and a peptide synthesizer (Model  
430A, Applied Biosystems Inc.).

15 A starting amino acid, Boc-Leu, was activated with  
HOBt/DCC and then condensed to the resin. Thereafter, the  
Boc group on the resin was treated with 50% trifluoroacetic  
acid/methylene chloride to deprotect the amino group. To  
this free amino group, the following protected amino acids  
20 activated with HOBt/DCC were condensed in turn according to  
the amino acid sequence of [Cys<sup>10</sup>]PACAP(11-27) NH<sub>2</sub>:

Boc-Val, Boc-Ala, Boc-Tyr(Br-Z), Boc-Lys(Cl-Z),

Boc-Met, Boc-Gln, Boc-Arg(Tos), Boc-Ser(Bzl),

Boc-Cys(MeBzl)

25 After the additional condensation by the amino acids  
activated by DCC or HOBt/DCC, the unreacted amino groups  
were acetylated with acetic anhydride to obtain 1.20 g of a

- 42 -

protected [Cys<sup>10</sup>]PACAP(11-27) NH<sub>2</sub> resin.

0.60 g of the resulting protected resin was treated with 10 ml of absolute hydrogen fluoride in the presence of 1.0 g of p-cresol at 0°C for 60 minutes, followed by removal  
5 of excess hydrogen fluoride by distillation under reduced pressure. The residue was washed twice with 5 ml of ethyl ether, and then extracted with 5 ml of 50% aqueous acetic acid. The insoluble material was removed by filtration and washed with 5 ml of 50% aqueous acetic acid. The filtrate  
10 and the washings were combined, and the combined solution was concentrated to 2 to 3 ml. The concentrated solution was applied on a Sephadex LH-20 column (2 X 75 cm) for elution with 50% acetic acid. The main fractions were collected, followed by distillation under reduced pressure.  
15 The residue was dissolved in 100 ml of 0.1% aqueous trifluoroacetic acid. The resulting solution was subjected to a YMC-ODS AM120 S-50 resin column (2.6 X 7 cm) and eluted by a liner gradient of 0.1% aqueous trifluoroacetic acid and 50% acetonitrile containing 0.1% trifluoroacetic acid. The  
20 main fractions were collected, followed by lyophilization. Thus, 70 mg of white powder was obtained.

Anal. for amino acids:

Ser 0.92(1), Glu 1.07(1), Ala 2.00(2), Val 1.96(2), Met  
0.88(1), Leu 1.93(2), Tyr 1.87(2), Lys 2.91(3), Arg  
25 2.17(2)

(M + H)<sup>+</sup> by mass spectrography (SIMS): 2127.9

HPLS elution time: 20.8 minutes

- 43 -

## Column conditions

Column: YMC-ODS (AM-301, S-5 120A)

Eluent: A (0.1% aqueous trifluoroacetic acid)

B (acetonitrile containing 0.1% trifluoroacetic  
acid)A linear gradient elution from the eluent A to the  
eluent B for 50 minutes

Flow rate: 1.0 ml/minute

Example 2-1 Preparation of Immunogen Containing PACAP38NH<sub>2</sub>

10 A complex comprising PACAP38NH<sub>2</sub> obtained in Reference  
Example 1 described above and bovine thyroglobulin  
(hereinafter referred to as BTG) was prepared, and it was  
used as an immunogen.

15 Namely, 2.8 mg of PACAP38NH<sub>2</sub> and 8.4 mg of BTG were  
dissolved in 1 ml of 0.1 M phosphate buffer (pH 6.9), and  
glutaraldehyde was added thereto to a final concentration of  
0.04%, followed by reaction at room temperature for 2 hours.

After reaction, the resulting product was dialyzed against  
physiological saline at 4°C for 2 days.

20 Example 2-2 Preparation of Immunogen Containing PACAP(11-  
27)NH<sub>2</sub>

A complex comprising [Cys<sup>10</sup>]PACAP(11-27)NH<sub>2</sub> obtained in  
Example 1-5 described above and BTG was prepared, and it was  
used as an immunogen.

25 Namely, 20 mg of BTG was dissolved in 1.4 ml of 0.1 M  
phosphate buffer (pH 6.9). The resulting solution was mixed  
with 100 µl of a DMF solution containing 2.2 mg (8 µmoles)

- 44 -

of N-( $\gamma$ -maleimidobutyryloxy)succinimide (hereinafter referred to as GMBS), followed by reaction at room temperature for 40 minutes. After reaction, the resulting product was fractionated on a Sephadex G-25 column, thereby  
5 obtaining a maleimide group-introduced BTG. Then, 12 mg of the maleimide group introduced BTG and 3.0 mg of [Cys<sup>10</sup>]PACAP(11-27)NH<sub>2</sub> were mixed and reacted with each other at 4°C for 3 days. After reaction, the reaction product was dialyzed against physiological saline at 4°C for  
10 2 days.

Example 3-1 Immunization of PACAP38NH<sub>2</sub>-BTG Conjugate

The female mice BALB/C 6 to 8 weeks old were subcutaneously immunized with 80  $\mu$ g/mouse of the immunogen PACAP38NH<sub>2</sub>-BTG complex, obtained in Example 2-1 described  
15 above, together with Freund's complete adjuvant. Then, the mice were additionally immunized with the same amount of the immunogen, together with Freund's incomplete adjuvant, 2 to 3 times at 4-week intervals.

Example 3-2 Immunization of PACAP(11-27)NH<sub>2</sub>-BTG Conjugate

20 The male rabbits were subcutaneously immunized with 400  $\mu$ g/rabbit of the PACAP(11-27)NH<sub>2</sub>-BTG complex, obtained in Example 2-2 described above, together with Freund's complete adjuvant. Then, the rabbits were additionally immunized with the same amount of the immunogen, together with  
25 Freund's incomplete adjuvant, 6 times at 4-week intervals.

Example 4-1 Preparation of Horseradish Peroxidase (HRP)-Labeled PACAP38NH<sub>2</sub>

- 45 -

A marker for the enzyme immunoassay (EIA) was prepared by crosslinking PACAP38NH<sub>2</sub> obtained in Reference Example 1 and HRP.

Namely, 180 nmoles of PACAP38NH<sub>2</sub> was dissolved in 500  
5 µl of 0.1 M phosphate buffer (pH 6.8), and 50 µl of a DMF solution containing 450 nmoles of GMBS was mixed therewith, followed by reaction at room temperature for 30 minutes. After reaction, the resulting product was fractionated on a Sephadex G-15 column. Thus, 100 nmoles of a maleimide  
10 group-introduced polypeptide was obtained.

On the other hand, 7.9 mg (200 nmoles) of HRP was dissolved in 0.95 ml of 0.02 M phosphate buffer (pH 6.8) containing 0.15 M NaCl, and 50 µl of a DMF solution containing 1.54 mg (4.9 µmoles) of N-succinimidyl-3-(2-  
15 pyridylthio)propionate (hereinafter referred as SPDP) was mixed therewith, followed by reaction at room temperature for 40 minutes. After reaction, 0.33 ml of 0.1 M acetate buffer (pH 4.5) containing 8.2 mg (53 µmoles) of  
20 dithiothreitol was added thereto, followed by reaction at room temperature for 20 minutes. Then, the reaction product was fractionated on a Sephadex G-25 column. Thus, 6 mg (100 nmoles) of a SH group-introduced enzyme was obtained.

Then, 100 nmoles of maleimide group-introduced PACAP38NH<sub>2</sub> and 100 nmoles of SH group-introduced HRP were  
25 mixed and reacted with each other at 4°C for 16 hours. After reaction, the reaction product was fractionated on an Ultrogel AcA44 (LKB-Pharmacia) to obtain HRP-labeled

PACAP38NH<sub>2</sub>.

Example 4-2 Preparation of HRP-Labeled PACAP(11-27)NH<sub>2</sub>

In 950  $\mu$ l of 180 nmoles of 0.1 M phosphate buffer (pH 6.8) was dissolved 8 mg (200 nmoles) of HRP, and 50  $\mu$ l of a DMF solution containing 1.4 mg (5  $\mu$ moles) of GMBS was mixed therewith, followed by reaction at room temperature for 40 minutes. Thereafter, the resulting product was fractionated on a Sephadex G-15 column to obtain maleimide group-introduced HRP. Then, 3.3 mg (78 nmoles) of maleimide group-introduced HRP thus prepared and 0.65 mg (310 nmoles) of [Cys<sup>10</sup>]PACAP(11-27)NH<sub>2</sub> prepared in Example 1-5 were mixed and reacted with each other at 4°C for 1 day. After reaction, the reaction product was fractionated on an Ultrogel AcA44 (LKB-Pharmacia) to obtain HRP-labeled [Cys<sup>10</sup>]PACAP(11-27)NH<sub>2</sub>.

Example 5-1 Determination of Antibody Titer of Mouse Antiserum

The antibody titer of the mouse antiserum was determined by the following method. In order to prepare an anti-mouse immunoglobulin antibody-bound microplate, 100  $\mu$ l of 0.1 M carbonate buffer (pH 9.6) containing 100  $\mu$ g/ml of the anti-mouse immunoglobulin antibody [IgG fraction, Kappel) was first poured into each well of a 96-well microplate, and the plate was allowed to stand at 4°C for 24 hours. After the plate was washed with phosphate buffered saline (hereinafter referred to as PBS), 300  $\mu$ l of PBS containing 25% Blockace (Snow Brand Milk Products) was



- 47 -

poured into each well to block excess binding sites of the wells, and treated at a temperature of at least 4°C for 24 hours.

To each well of the above-mentioned anti-mouse  
5 immunoglobulin antibody-bound microplate were added 50 µl of  
buffer E [0.02 M phosphate buffer (pH 7.0) containing 10%  
Blockace, 2 mg/ml bovine serum albumin (hereinafter referred  
to as BSA), 0.4 M NaCl, 2mM EDTA and 0.1% NaN<sub>3</sub>] and 50 µl of  
the mouse anti-PACAP38NH<sub>2</sub> antiserum diluted with buffer E,  
10 followed by reaction at 4°C for 16 hours. After the plate  
was washed with PBS, 100 µl of the HRP-labeled PACAP38NH<sub>2</sub>  
prepared in Example 4-1 described above [diluted 100 times  
with buffer H (pH 7.0) containing 2 mg/ml BSA and 0.15 M  
NaCl was added to each well, followed by reaction at room  
15 temperature for 6 hours. After reaction, the plate was  
washed with PBS, and then 100 µl of 0.1 M citrate buffer (pH  
5.5) containing 0.2% o-phenylenediamine and 0.02% hydrogen  
peroxide was poured into each well to assay the enzyme  
activity on the solid phase, followed by reaction at room  
20 temperature for 10 minutes. After 100 µl of 4 N sulfuric  
acid was added thereto to terminate the reaction, the  
absorption at 492 nm was measured by a plate reader (MTP-32,  
Corona).

The results are shown in Fig. 1. Increases in anti-  
25 PACAP38 antibody titer were observed in 4 mice of the 8  
immunized mice.

Example 5-2 Determination of Antibody Titer of Rabbit Antiserum

The antibody titer of the rabbit antiserum was determined in a similar manner. An anti-rabbit immunoglobulin antibody (IgG fraction, Kappel)-bound microplate was prepared in the same manner as with Example 5-1 described above. To each well of the above plate were added 50  $\mu$ l of buffer E and 50  $\mu$ l of the rabbit anti-PACAP(11-27)NH<sub>2</sub> antiserum diluted with buffer E, followed by reaction at 4°C for 16 hours. After the plate was washed with PBS, 100  $\mu$ l of HRP-labeled PACAP(11-27)NH<sub>2</sub> prepared in Example 4-2 described above [diluted 200 times with buffer E] was added to each well, followed by reaction at room temperature for 6 hours. After reaction, the plate was washed with PBS, and then the enzyme activity on the solid phase was assayed by the use of the TMB microwell peroxidase substrate system (Kirkegaard & Perry Lab., Inc., sold by Funakoshi Yakuhin). A plate reader (MTP-32, Corona) was used for measurement of the absorbance (at 450 nm).

The results are shown in Fig. 2. High antibody titer was detected in all of the immunized rabbits.

Example 6 Cell Fusion

Mouse No. 5 which showed relatively high antibody titer was inoculated with a solution prepared by dissolving 200  $\mu$ g of the immunogen in 0.25 ml of physiological saline to conduct the final immunization. The spleen was taken out of the mouse after 3 days from the final immunization, pressed

- 49 -

by a stainless mesh, filtered, and floated in Eagle's minimum essential medium (MEM), thereby obtaining a spleen cell-floating solution. As a cell for cell fusion, BALB/C mouse-derived myeroma cell P3-X63.Ag8.U1 (P3U1) was used

5 [Current Topics in Microbiology and Immunology 81, 1  
(1978)]. The cell fusion was carried out in accordance with the original method [Nature 256, 495 (1957)]. Namely, the spleen cells and the P3U1 cells were washed 3 times with serum-free MEM, and mixed with each other so that the number

10 ratio of the spleen cells to the P3U1 cells reached 5:1. The mixture was centrifuged at 800 rpm for 15 minutes to precipitate the cells. After the supernatant was thoroughly removed, the precipitate was lightly loosened, and 0.3 ml of 45% polyethylene glycol (PEG) 600 (Kochlight) was added

15 thereto. Then, the mixture was allowed to stand in a hot water bath at 37°C for 7 minutes to perform the fusion. After the fusion was completed, MEM was added to the cells at a rate of 2 ml per minute. After 12 ml of MEM was added in total, the supernatant was removed by centrifugation at

20 600 rpm for 15 minutes. The resulting precipitate was floated in GIT medium (GIT-10FCS, Wako Pure Chemical Industries) containing 10% fetal calf serum so that the P3U1 cells were contained in an amount of  $2 \times 10^6$  cells/ml. The resulting suspension was seeded in 120 wells of a 24-well

25 multi-dish (Linbro) in an amount of 1 ml/well. After seeding, the cells were incubated in a 5% carbon dioxide incubator at 37°C. After 24 hours, 1 ml of GIT-10FCS medium

(HAT medium) containing HAT ( $1 \times 10^{-4}$  M hypoxanthine,  $4 \times 10^{-7}$  M aminopterin and  $1.6 \times 10^{-3}$  M thymidine) was added to each well to initiate HAT selective culture. The HAT selective culture was continued by discarding 1 ml of old liquor and then supplying 1 ml of HAT medium, 3, 6 and 9 days after the initiation of the culture. The proliferation of hybridoma cells was observed 9 to 14 days after the completion of the cell fusion. When the culture solution turned yellow (about  $1 \times 10^5$  cells/ml), the supernatants were recovered and the antibody titer was assayed.

#### Example 7 Screening of Hybridoma Cell

To the anti-mouse immunoglobulin antibody-bound microplate were added 50  $\mu$ l of buffer E and 50  $\mu$ l of the hybridoma culture supernatant, followed by reaction at room temperature for 6 hours. After the plate was washed with PBS, 100  $\mu$ l of the HRP-labeled PACAP38NH<sub>2</sub> prepared in Example 4 described above (diluted 200 times with buffer H) was added thereto, followed by reaction at 4°C for 16 hours.

After the plate was washed with PBS, the enzyme activity on the solid phase was assayed by the method described in Example 5-1 described above.

The supernatants of all of the 120 wells in which the proliferation of the cells was observed were thus examined. As a result, antibody titer was detected in 18 wells.

#### Example 8 Cloning

Of the wells which showed positive antibody activity, the hybridoma cells contained in the wells of Nos. 44, 49,

- 51 -

97 and 113 were cloned by the limiting dilution method. Namely, the hybridoma cells were floated in RPMI 1640-20FCS so as to be contained in an amount of 1.5 cells/ml, and 2.0 ml thereof was poured into each well of a 96-well microplate (Nunk). In pouring, the thymocytes of BALB/C mice were added thereto as feeder cells so as to be contained in an amount of  $5 \times 10^5$  cells/well. After about one week, the proliferation of the cells was observed. The antibody titer of the supernatants was examined by the EIA described in

Example 5. As a result, antibodies were produced in 28 clones of 30 clones for the hybridoma cells of No. 44, in 47 clones of 50 clones for the hybridoma cells of No. 49, in 49 clones of 50 clones for the hybridoma cells of No. 97 and in 48 clones of 50 clones for the hybridoma cells of No. 113. Of these clones, giving attention to clone PA-6N obtained from No. 44-2 and monoclonal antibody PA-6Na produced thereby, clone PA-1N obtained from No. 49-3 and monoclonal antibody PA-1Na produced thereby, clone PA-2C obtained from No. 97-2 and monoclonal antibody PA-2Ca produced thereby, and clone PA-5N obtained from No. 113-5 and monoclonal antibody PA-5Na produced thereby, the following experiments were made.

Similarly, the cell fusion experiment was conducted using the spleen cells of another mouse during immunization.

Giving attention also to clone PA-1C obtained from No. 28-12 and monoclonal antibody PA-1Ca produced thereby, and clone PA-3N obtained from No. 10-3 and monoclonal antibody

PA-3Na produced thereby, the following experiment was carried out.

Example 9 Preparation of Large Amount of Monoclonal Antibodies

5       The mice which had been given 0.5 ml of mineral oil intraperitoneally or the untreated mice (BALB/C) were injected intraperitoneally with 1 to  $3 \times 10^5$  cells/mouse of the above-mentioned hybridoma cells, and then the antibody-containing ascites was recovered after 6 to 20 days.

10      Example 10 Purification of Monoclonal Antibodies

      The monoclonal antibodies were purified by a protein-A column or a diethylaminoethyl (DEAE)-cellulose column from the ascites obtained in Example 9 described above.

      Namely, 6 ml of the ascites containing PA-1N was  
15   diluted with the same amount of a binding buffer (pH 9.0, 1.5 M glycine containing 3.5 M NaCl and 0.05%  $\text{NaN}_3$ ). The resulting solution was subjected to a protein-A Sepharose (Pharmacia) column which had been pre-equilibrated with the binding buffer, and a specific antibody was eluted with an  
20   eluting buffer (pH 3.0, 0.1 M citrate buffer containing 0.05%  $\text{NaN}_3$ ). By the above procedures, 28 mg of the specific antibody was obtained.

      Similarly, 23 mg of a specific antibody was obtained from 5 ml of the ascites containing PA-5N, 13 mg of a  
25   specific antibody was obtained from 7.5 ml of the ascites containing PA-6N, and 45 mg of a specific antibody was obtained from 14 ml of the ascites containing PA-1C.

On the other hand, a saturated ammonium sulfate solution was added to 20 ml of the ascites containing PA-3N to a final concentration of 45% for salt precipitation, followed by centrifugation (20,000 g, 30 minutes). The precipitate fraction was dialyzed against 0.02 M borate buffer (pH 7) containing 0.15 M NaCl (hereinafter referred to as BBS), and further dialyzed against 0.01 M phosphate buffer containing 0.01 M NaCl. The antibody fraction was loaded on a DEAE cellulose column (DE-52, Wattman, 2.5 cm in diameter X 10 cm), and eluted by a linear concentration gradient (0.01 M-0.35 M) of 100 ml of NaCl. By the above procedures, 136 mg of a specific antibody was obtained.

Similarly, 57 mg of a specific antibody was obtained from 7.5 ml of the ascites containing PA-2C.

Example 11 Determination of Class and Subclass of Monoclonal Antibody

Into each well of a 96-well microplate was poured 100  $\mu$ l of 0.1 M carbonate buffer (pH 9.6) containing 5  $\mu$ g/ml of PACAP38NH<sub>2</sub> prepared above, and the microplate was allowed to stand at 4°C for 24 hours. The excess binding sites of the wells were blocked with Blockace according to the method described in Example 5-1 to prepare a PACAP38 NH<sub>2</sub>-bound plate. Then, each of supernatants of PA-1N, PA-3N, PA-5N, PA-6N, PA-2C and PA-1C was added to each well of the plate in an amount of 100  $\mu$ l, followed by reaction at room temperature for 3 hours. Then, the class and subclass were examined by the enzyme-linked immunosorbent assay (ELISA)

using an isotype typing kit (Mouse-Typer<sup>TM</sup> Sub-Isotyping Kit, Bio RAD). As a result, PA-1Na, PA-6Na, PA-2Ca and PA-1Ca belonged to IgG1,  $\kappa$ , PA-5Na belonged to IgG2a,  $\kappa$ , and PA-3Na belonged to IgG2b,  $\kappa$ .

5 Example 12 Preparation of F(ab')<sub>2</sub> Fraction

PA-6Na described in Example 10 was concentrated to 8 mg/500  $\mu$ l by a Collodion bag (Emuesu Kiki), and then dialyzed against 0.1 M acetate buffer containing 0.1 M NaCl.

To the resulting antibody solution was added 0.4 mg of  
10 pepsin (crystallized twice, Sigma), followed by reaction at 37°C for 16 hours. Then, the F(ab')<sub>2</sub> fraction was purified by an FPLC (Pharmacia) using a Superrose 12 column equilibrated with 0.1 M phosphate buffer (pH 6.8).

By a similar technique, 0.445 mg of pepsin was added to  
15 8.9 mg of PA-1Ca described in Example 10 to prepare the F(ab') fraction.

Example 13 Preparation of HRP-Labeled Anti-PACAP Monoclonal Antibodies

(1) PA-6Na F(ab')<sub>2</sub>-HRP

20 To 1 ml of a solution containing 2.2 mg (22 nmoles)/ml of the PA-6Na F(ab')<sub>2</sub> fraction described in Example 12 was added 50  $\mu$ l of a DMF solution containing 260 nmoles of GMBS, followed by reaction at room temperature for 40 minutes. The reaction solution was fractionated on a Sephadex G-25  
25 column [1 cm in diameter X 30 cm, eluent: 0.1 M phosphate buffer (pH 6.7)] to obtain a maleimide group-introduced F(ab')<sub>2</sub> fraction. With 1.5 mg of the resulting F(ab')<sub>2</sub>



- 55 -

fraction was mixed 5.5 mg of SH group-introduced HRP prepared by the method described in Example 4-1, and the reaction product was concentrated to about 0.3 ml by a collodion bag, followed by standing at 4°C for 16 hours.

- 5 The reaction solution was loaded on an Ultrogel ACA34 column (10 mm in diameter X 40 mm) to purify an  $F(ab')_2$ -HRP complex fraction. It was confirmed from the absorbance at 280 nm and 403 nm that HRP was introduced in an amount of 2.4 molecules/molecule of  $F(ab')_2$ .

10 (2) PA-1Ca  $F(ab')_2$ -HRP

In a similar manner, an  $F(ab')_2$ -HRP complex was prepared by using 2.9 mg of the PA-1Ca  $F(ab')_2$  fraction described in Example 12.

(3) PA-2Ca IgG-HRP

- 15 To 6.4 mg (43 nmoles) of the PA-2Ca purified fraction described in Example 10 was added 15-fold moles of GMBS, followed by introduction of a maleimide group. Then, the resulting product was reacted with SH group-introduced HRP in a similar manner to prepare a marker into which HRP was  
20 introduced in an amount of 2.4 molecules/molecule of IgG.

Example 14 Competitive Method-EIA

(1) Competitive Method-EIA Using PA-1Na

- To the anti-mouse immunoglobulin antibody-bound microplate described in Example 5 were added 50 µl of a PA-  
25 1N culture supernatant diluted 50 times with buffer H and 50 µl of a buffer H solution of PACAP or a partial peptide of PACAP, such as PACAP38NH<sub>2</sub>, PACAP27NH<sub>2</sub>, PACAP(4-27), PACAP(1-

- 56 -

13), PACAP(14-38)NH<sub>2</sub>, PACAP(31-38)NH<sub>2</sub> or VIP, followed by reaction at room temperature for 2 hours. Then, 50 µl of HRP-labeled PACAP38NH<sub>2</sub> described in Example 4-1 (diluted 100 times with buffer H) was added thereto, followed by reaction  
5 at 4°C for 16 hours. After reaction, the plate was washed with PBS, and then the enzyme activity on the solid phase was assayed by the method described in Example 5. The results are shown in Fig. 3(a). In the drawing, -o-, -●-, -▲-, -■-, -△-, -□- and -X- indicate PACAP38NH<sub>2</sub>, PACAP27NH<sub>2</sub>,  
10 PACAP(4-27)OH, PACAP(1-13)OH, PACAP(14-38)NH<sub>2</sub>, PACAP(31-38)NH<sub>2</sub> and a standard curve of VIP, respectively.

As shown in Fig. 3(a), PA-1Na reacts with PACAP38NH<sub>2</sub>, PACAP27NH<sub>2</sub>, PACAP(1-13)OH and PACAP(4-27)OH, but does not react with PACAP(14-38)NH<sub>2</sub> and PACAP(31-38)NH<sub>2</sub>. PA-1Na does  
15 not react with VIP either (the cross reactivity to PACAP38NH<sub>2</sub> is 0.1% or less). These results reveal that PA-1Na is an antibody belonging to class Ia which recognizes the N-terminal portion of PACAP38NH<sub>2</sub>.

#### (2) Competitive Method-EIA Using PA-5Na

20 A competitive method-EIA using PA-5Na was carried out by the method described described in Example 14-(1). A culture supernatant of PA-5N was diluted 70 times. The results are shown in Fig. 3(c). PA-5Na reacts with PACAP38NH<sub>2</sub>, PACAP27NH<sub>2</sub>, PACAP(1-13)OH and PACAP(4-27)OH, but  
25 does not react with PACAP(14-38)NH<sub>2</sub> and PACAP(31-38)NH<sub>2</sub>. PA-5Na does not react with VIP either (the cross reactivity is 0.1% or less). These results reveal that PA-5Na is an

antibody belonging to class Ia which recognizes the N-terminal portion of PACAP38NH<sub>2</sub>.

PA-1Na is different from PA-5Na in cross reactivity (to PACAP38NH<sub>2</sub>) with PACAP(1-13)OH, and the cross reactivity of  
5 the former is at least 10 times stronger than that of the latter.

### (3) Competitive Method-EIA Using PA-3Na

A competitive method-EIA using PA-3Na was carried out by the method described in Example 14-(1). A culture  
10 supernatant of PA-3N was diluted 50 times. The results are shown in Fig. 3(b). PA-3Na reacts with PACAP38NH<sub>2</sub>, PACAP27NH<sub>2</sub>, PACAP(1-13)OH and PACAP(4-27)OH, but does not react with PACAP(14-38)NH<sub>2</sub> and PACAP(31-38)NH<sub>2</sub>. On the  
other hand, PA-3Na shows a cross reactivity of 1% with VIP  
15 (to PACAP38NH<sub>2</sub>). These results reveal that PA-5Na is an antibody belonging to class Ib which recognizes the N-terminal portion of PACAP38NH<sub>2</sub>.

### (4) Competitive Method-EIA Using PA-6Na

A competitive method-EIA using PA-6Na was carried out  
20 by the method described in Example 14-(1). A culture supernatant of PA-6N was diluted 40 times. The results are shown in Fig. 3(d). PA-6Na reacts with PACAP38NH<sub>2</sub>, PACAP27NH<sub>2</sub> and PACAP(4-27)OH, but does not react with  
PACAP(1-13)OH, PACAP(14-38)NH<sub>2</sub> and PACAP(31-38)NH<sub>2</sub>. PA-6Na  
25 does not react with VIP either (the cross reactivity to PACAP38NH<sub>2</sub> is 0.1% or less). These results reveal that PA-6Na is an antibody belonging to class II which recognizes

the region from the N-terminal portion to the central portion of PACAP38NH<sub>2</sub>.

(5) Competitive Method-EIA Using PA-2Ca

A competitive method-EIA using PA-2Ca was carried out by the method described described in Example 14-(1). A culture supernatant of PA-2C was diluted 340 times. The results are shown in Fig. 3(e). PA-1Ca reacts with PACAP38NH<sub>2</sub> and PACAP(14-38)NH<sub>2</sub>, but does not react with PACAP27NH<sub>2</sub>, PACAP(4-27)OH PACAP(1-13)OH and PACAP(31-38)NH<sub>2</sub>. PA-2Ca does not react with VIP either (the cross reactivity to PACAP38NH<sub>2</sub> is 0.1% or less). These results reveal that PA-6Na is an antibody belonging to class III which recognizes the region from the C-terminal portion to the central portion of PACAP38NH<sub>2</sub>.

(6) Competitive Method-EIA Using PA-1Ca

A competitive method-EIA using PA-1Ca was carried out by the method described described in Example 14-(1). A culture supernatant of PA-1C was diluted 35 times. The results are shown in Fig. 3(f). PA-1Ca reacts with PACAP38NH<sub>2</sub>, PACAP(14-38)NH<sub>2</sub> AND pacap(31-38)NH<sub>2</sub>, but does not react with PACAP27NH<sub>2</sub>, PACAP(4-27)OH and PACAP(1-13)OH. PA-6Na does not react with VIP either (the cross reactivity to PACAP38NH<sub>2</sub> is 0.1% or less). These results reveal that PA-6Na is an antibody belonging to class IV which recognizes the C-terminal portion of PACAP38NH<sub>2</sub>.

At least 400, 100, 200, 20 or 200 pg/well (a PACAP concentration giving B/BO = 80%) of PACAP38NH<sub>2</sub> could be

detected by the competitive method EIA using PA-1Na, PA-5Na, PA-3Na, PA-6Na, PA-2Ca or PA-1Ca.

Example 15 Sandwich Method-EIA

(1) Sandwich Method-EIA Using PA-6Na F(ab')<sub>2</sub>-HRP

5 Into each well of a 96-well microplate was poured 100  $\mu$ l of 0.1 M carbonate buffer (pH 9.6) containing 15  $\mu$ g/ml of purified monoclonal antibody PA-1Na, PA-3Na, PA-5Na, PA-6Na, PA-2Ca or PA-1Ca described in Example 10, and the plate was allowed to stand at 4°C for 24 hours. The excess binding  
10 sites of the wells were inactivated by adding 300  $\mu$ l of Blockace diluted 4 times with PBS.

To each well of the plate thus prepared was added 100  $\mu$ l of a standard solution of PACAP38NH<sub>2</sub> diluted with buffer E, followed by reaction at 4°C for 24 hours. After washing  
15 with PBS, 100  $\mu$ l of HRP-labeled PA- $\gamma$ Na F(ab')<sub>2</sub> prepared in Example 13-(1) described above (diluted 100 times with buffer C) was added thereto, followed by reaction at 4°C for 24 hours. After washing with PBS, the enzyme activity on the solid phase was assayed by the method described in  
20 Example 5. The results are shown in Fig. 4.

In the sandwich method-EIA using PA-6Na (class II) F(ab')<sub>2</sub>-HRP, the sensitivity was highest when PA-2Ca (class III) was used as the antibody for solid phase, and at least 0.4 pg/well of PACAP38NH<sub>2</sub> could be detected. When PA-1Ca  
25 (class IV) was used as the antibody for solid phase, 2 pg/well of PACAP38NH<sub>2</sub> could be detected, and when PA-5Na (class Ia) was used, 40 pg/well of PACAP38NH<sub>2</sub> could be

- 60 -

detected.

The above results reveal that when PA-6Na, an antibody of class II, is used as a marker, the sandwich-EIA can be established even if an antibody of any other class

- 5 (including a class adjacent thereto in primary arrangement) is used as the antibody for solid phase, and particularly that the sandwich method-EIA using PA-2Ca, an antibody of class III, as the antibody for solid phase is highly sensitive.

10 (2) Sandwich Method-EIA Using PA-1Ca  $F(ab')_2$ -HRP

- A sandwich method-EIA using a microplate sensitized with each of various antibodies described in the above item (1) and HRP-labeled PA-1Ca  $F(ab')_2$  described in Example 13- (2) was carried out by the method described in the above  
15 item (1). The results are shown in Fig. 5.

In the sandwich method-EIA using PA-1Ca (class IV)  $F(ab')_2$ -HRP, the sensitivity was highest when PA-3Na (class Ib) was used as the antibody for solid phase, and at least 1 pg/well of PACAP38NH<sub>2</sub> could be detected. When PA-1Na (class  
20 Ia), PA-5Na (class Ia) or PA-6Na (class II) was used as the antibody for solid phase, at least 2 pg/well of PACAP38NH<sub>2</sub> could be detected. Further, even when an antibody of class III adjacent in primary arrangement was used, at least 8 pg/well of PACAP38NH<sub>2</sub> was detected.

- 25 The above results reveal that when PA-1Ca, an antibody of class IV, is used as a marker, the sandwich-EIA can be established even if an antibody of any other class

(including a class adjacent thereto in primary arrangement) is used as the antibody for solid phase, and particularly that the sandwich method-EIA using PA-3Na, an antibody of class Ib, as the antibody for solid phase is highly  
5 sensitive.

(3) Sandwich Method-EIA Using PA-2Ca IgG-HRP

A sandwich method-EIA using a microplate sensitized with each of various antibodies described in the above item (1) and HRP-labeled PA-2Ca IgG described in Example 13-  
10 (3) was carried out by the method described in the above item (1). The results are shown in Fig. 6.

In the sandwich method-EIA using PA-2Ca (class III) IgG-HRP, the sensitivity was highest when PA-3Na (class Ib) was used as the antibody for solid phase, and at least 2  
15 pg/well of PACAP38NH<sub>2</sub> could be detected. When PA-1Na, PA-5Na or PA-6Na was used as the antibody for solid phase, 4 pg/well of PACAP38NH<sub>2</sub> was detected, and when PA-1Ca was used, 80 pg/well of PACAP38NH<sub>2</sub> was detected.

The above results reveal that when PA-2Ca, an antibody  
20 of class III, is used as a marker, the sandwich-EIA can be established even if an antibody of any other class (including a class adjacent thereto in primary arrangement) is used as the antibody for solid phase, and particularly that the sandwich method-EIA using PA-3Na, an antibody of  
25 class Ib, as the antibody for solid phase is highly sensitive.

Example 16 Specificity of Sandwich Method-EIA

In a sandwich method-EIA using PA-2Ca described in Example 15-(1) as the antibody for solid phase and PA-6Na F(ab')<sub>2</sub>-HRP as the antibody for labeling, the reactivity to PACAP38NH<sub>2</sub>, VIP GRF and secretin was examined. The results are shown in Fig. 7.

In the drawing, -o-, -●-, -▲-, -△- and -■- indicate concentration-dependent curves of PACAP38NH<sub>2</sub>, PACAP27NH<sub>2</sub>, VIP, GRF and secretin, respectively.

10 In the above assay, the cross reactivity to all of PACAP27NH<sub>2</sub>, VIP, GRF and secretin is 0.001%. This reveals that the above assay is specific for PACAP38NH<sub>2</sub>.

Example 17 Purification of Rabbit PACAP(11-27)NH<sub>2</sub> Antibody

15 An affinity solid phase for purifying rabbit PACAP(11-27)NH<sub>2</sub> was prepared. Namely, 4.5 mg of [Cys<sup>10</sup>]PACAP(11-27)NH<sub>2</sub> was dissolved in 20 ml of 0.1 M sodium hydrogencarbonate containing 0.5 M NaCl, and reacted with 3 g of CNBr-activated Sepharose 4B at room temperature for 3 hours. Then, after unreacted active groups were treated  
20 with 0.1 M Tris-hydrochloric acid buffer (pH 8), the resulting product was dispersed in PBS and charged into a column.

8 ml of rabbit PACAP(11-27)NH<sub>2</sub> antiserum 1C, 8 ml of 2C and 16ml of 3C in which high antibody activity was observed  
25 (see Fig. 2) were mixed with one another, and 32 ml of PBS was added thereto. Then, 52 ml of saturated ammonium sulfate was slowly added thereto with stirring, followed by



- 63 -

centrifugation at 12,000 X g for 20 minutes. The precipitate was dissolved in 25 ml of borate buffer (pH 8) containing 0.15 M NaCl (BBS), followed by dialysis against BBS at 4°C for 2 days. After dialysis, the resulting  
5 solution was loaded on the above-mentioned column and thoroughly washed with BBS. Then, specific antibodies were eluted with 0.1 M acetate buffer (pH 4.5) containing 0.5 M NaCl and further with 0.05 M glycine-hydrochloric acid buffer (pH 2.0) containing 0.1 M NaCl. As a result, 5.4 mg  
10 and 6.7 mg of the specific antibodies were obtained in fractions eluted at pH 4 and pH 2, respectively.

Example 18 Preparation of Anti-PACAP(11-27)NH<sub>2</sub> Fab'-HRP

An Fab'-peroxidase marker was prepared from the anti-PACAP(11-27)NH<sub>2</sub> antibody described in Example 17 according  
15 to the method of Ishikawa et al. [J. Appl. Biochem. 6, 56-63 (1984)].

Namely, 5.6 mg of the specific antibody was dissolved in 0.1 M acetate buffer (pH 4.5), and 160 µg of pepsin (crystallized twice, Sigma) was added thereto, followed by  
20 reaction at 37°C for 20 hours. Then, the reaction product was subjected to an FPLC (Pharmacia) using a Superose 12 column equilibrated with 0.1 M acetate buffer (pH 5) to obtain 2.2 mg of an F(ab')<sub>2</sub> fraction. β-Mercaptoethylamine was added to this fraction to a final concentration of 20  
25 mM, and the resulting solution was allowed to stand at 37°C for 90 minutes. Then, the reaction solution was separated on a Sephadex G-25 Column equilibrated with 0.1 M phosphate

- 64 -

buffer (pH 6.0) containing 5 mM EDTA to obtain an Fab' fraction.

On the other hand, 6 mg of maleimidated HRP prepared according to the method described in Example 4-2 and the total amount of the above-mentioned anti-PACAP(11-27)NH<sub>2</sub> Fab' fraction were mixed and reacted with each other at 4°C for 1 days. Then, the reaction product was fractionated on an Ultrogel AcA44 column equilibrated with 0.1 M phosphate buffer to purify HRP-labeled anti-PACAP(11-27)NH<sub>2</sub> Fab'.

10 Example 19 Sandwich Method-EIA for Assaying PACAP27NH<sub>2</sub>

(1) To a microplate on which PA-1Na described in Example 15 was fixed, 100 µl of a standard solution of PACAP27NH<sub>2</sub>, PACAP(11-27)OH or PACAP38NH<sub>2</sub> was added, followed by reaction at 4°C for 24 hours. After washing with PBS, HRP-labeled anti-PACAP(11-27)NH<sub>2</sub> Fab' described in Example 18 (diluted 400 times with buffer C) was added thereto and reacted at 4°C for 24 hours. After washing with PBS, the enzyme activity on the solid phase was assayed by the method described in Example 5-2. The results are shown in Fig. 8.

20 In the drawing, -●-, -▲-, -■- and -○- indicate PACAP27NH<sub>2</sub>, PACAP(11-27)OH, PACAP38NH<sub>2</sub> and VIP, respectively. The results shown in Fig. 8 reveal that 0.2 pg/well of PACAP27NH<sub>2</sub> can be detected by this assay with a cross reactivity of 11.5% with PACAP(11-27)OH and with a cross reactivity of 0.97% by weight ratio or 1.3% by mole ratio with PACAP38NH<sub>2</sub>.

- 65 -

(2) Using a microplate on which PA-3Na described in Example 15 was fixed and HRP-labeled PACAP(11-27)NH<sub>2</sub> Fab' described in Example 18, a sandwich method-EIA was carried out by the method described above. The results are shown in Fig. 9. These results reveal that 0.2 pg/well of PACAP27NH<sub>2</sub> can be detected by this assay with a cross reactivity of 11% with PACAP(11-27)OH and with a cross reactivity of 0.22% by weight ratio or 0.31% by mole ratio with PACAP38NH<sub>2</sub>.

(3) Using a microplate on which PA-5Na described in Example 15 was fixed and HRP-labeled PACAP(11-27)NH<sub>2</sub> Fab' described in Example 18, a sandwich method-EIA was carried out by the method described above. The results are shown in Fig. 10. These results reveal that 0.2 pg/well of PACAP27NH<sub>2</sub> can be detected by this assay with a cross reactivity of 4.1% with PACAP(11-27)OH and with a cross reactivity of 0.40% by weight ratio or 0.56% by mole ratio with PACAP38NH<sub>2</sub>.

(4) Using a microplate on which PA-6Na described in Example 15 was fixed and HRP-labeled PACAP(11-27)NH<sub>2</sub> Fab' described in Example 18, a sandwich method-EIA was carried out by the method described above. The results are shown in Fig. 11. These results reveal that 0.8 pg/well of PACAP27NH<sub>2</sub> can be detected by this assay with a cross reactivity of 5.4% with PACAP(11-27)OH and with a cross reactivity of 3.6% by weight ratio or 5.00% by mole ratio with PACAP38NH<sub>2</sub>.

All of the assays only exhibited a cross reactivity of

- 66 -

0.001% or less with VIP.

From the above results, PACAP27NH<sub>2</sub> can be detected by the use of these assays with a cross reactivity of 0.22 to 3.6% by weight ratio or with a cross reactivity of 0.31 to 5.0% by mole ratio with PACAP38NH<sub>2</sub>. It is therefore possible to fractionate and determine PACAP27NH<sub>2</sub> and PACAP38NH<sub>2</sub> by combinations of these assays and the assay described in Example 16.

10 Example 20 Examination of Neutralization Activity of Anti-PACAP Antibodies

Rat adrenal brown cytoma strain PC-12h (supplied by Dr. Hatanaka, Protein Laboratory, Osaka University) was disseminated at a rate of  $5 \times 10^4$  cells/well on a 48-well multi-well plate (Sumitomo Bakelite) treated with collagen, and incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS for 7 to 10 days. The medium of the plate was exchanged with Hnak's balanced salt solution (HBSS) containing 0.05% BSA, followed by incubation for 30 minutes. Then, PACAP38NH<sub>2</sub> (final concentration: 2 nM) was added thereto which had previously been reacted with each of the anti-PACAP antibodies (final concentration: 2, 20 or 200 nM) at 4°C for 1 hour. After additional incubation for 2 hours, the concentration of cAMP contained in the culture supernatant was measured with a cAMP measuring kit (Amersham). The results are shown in Fig. 12. In the drawing, -o-, -Δ-, -□-, -■-, -▲- and -●- indicate PA-1Na, PA-3Na, PA-5Na, PA-6Na, PA-2Ca and PA-1Ca, respectively.

These results reveal that four kinds of these six kinds of monoclonal anti-PACAP antibodies have neutralization activity to PACAP38NH<sub>2</sub> and the order of its strength is PA-2Ca (an antibody of class III) > PA-1Na (an antibody of class Ia) > PA-5Na (an antibody of class Ia) > PA-3Na (an antibody of class Ib).

The use of the various monoclonal antibodies of the present invention which recognized the continuous sites in primary arrangement of PACAP revealed immunochemical properties of PACAP. By using these antibodies, it is possible to establish assay systems using various competitive or sandwich methods different in specificity for PACAP or its related peptides. In particular, it becomes possible to fractionate and determine PACAP38NH<sub>2</sub> and PACAP27NH<sub>2</sub> with high sensitivity by combinations of various sandwich methods.

Example 21 Examination of Reactivity of Anti-PACAP Monoclonal Antibodies with Human PACAP Precursor

Escherichia coli cells having plasmid pTS401 in which human PACAP precursor genes were integrated were cultivated in 10 ml of M9 medium (containing 0.1% NZ amine, 0.4% glucose, 50 µg/ml ampicillin and 25 µg/ml chloramphenicol) at 37°C. When the cells were proliferated to have an absorbance of 0.7 at 600 nm, isopropyl-β-D-thiogalactopyranoside was added thereto, and cultivation was further continued for 3 hours, followed by centrifugation at 5,000 g for 10 minutes to collect the cells. As a control, the

- 68 -

cells were collected just before addition of isopropyl- $\beta$ -D-thiogalactopyranoside.

To the cells, 1 ml of SDS-containing sample buffer of Laemmli was added, and the mixture was boiled at 100°C for 5 minutes, followed by electrophoresis on 16% polyacrylamide gel. The protein was electrically transferred to nitrocellulose filters by the western blotting method, and each of anti-PACAP mouse monoclonal antibodies PA-1Na, PA-1Ca and PA-2Ca was reacted with the protein on each filter. Then, the secondary antibody (anti-mouse IgG-peroxidase, Cappel.) was reacted therewith. The band of the desired protein was stained and fixed by a POD immunostain set (Wako Pure Chemical Industries). The bands reacting with anti-PACAP mouse monoclonal antibodies PA-1Na and PA-2Ca were observed in the vicinity of a molecular weight of about 18,000 daltons. The bands approximately agree with molecular weight of human PACAP precursor deduced from cDNA coding for human PACAP. This fact reveals that anti-PACAP mouse monoclonal antibodies PA-1Na and PA-2Ca react with human PACAP precursor.

CLAIMS

WHAT IS CLAIMED IS:

1. An antibody which specifically binds to PACAP or a precursor thereof.
2. An antibody in accordance with claim 1, in which said PACAP is PACAP38NH<sub>2</sub> consisting of an amino acid sequence represented by formula [1]:

His Ser Asp Gly Ile Phe Thr Asp Ser Tyr  
Ser Arg Tyr Arg Lys Gln Met Ala Val Lys  
Lys Tyr Leu Ala Ala Val Leu Gly Lys Arg  
Tyr Lys Gln Arg Val Lys Asn Lys-NH<sub>2</sub> [1].

3. An antibody in accordance with claim 1, in which said precursor of PACAP is a precursor of human PACAP consisting of an amino acid sequence represented by formula [2] or a portion thereof:

Met Thr Met Cys Ser Gly Ala Arg Leu  
Ala Leu Leu Val Tyr Gly Ile Ile Met His  
Ser Ser Val Tyr Ser Ser Pro Ala Ala Ala  
Gly Leu Arg Phe Pro Gly Ile Arg Pro Glu  
Glu Glu Ala Tyr Gly Glu Asp Gly Asn Pro  
Leu Pro Asp Phe Gly Gly Ser Glu Pro Pro  
Gly Ala Gly Ser Pro Ala Ser Ala Pro Arg  
Ala Ala Ala Ala Trp Tyr Arg Pro Ala Gly  
Arg Arg Asp Val Ala His Gly Ile Leu Asn  
Glu Ala Tyr Arg Lys Val Leu Asp Gln Leu  
Ser Ala Gly Lys His Leu Gln Ser Leu Val  
Ala Arg Gly Val Gly Gly Ser Leu Gly Gly

- 70 -

Gly Ala Gly Asp Asp Ala Glu Pro Leu Ser  
Lys Arg His Ser Asp Gly Ile Phe Thr Asp  
Ser Tyr Ser Arg Tyr Arg Lys Gln Met Ala  
Val Lys Lys Tyr Leu Ala Ala Val Leu Gly  
Lys Arg Tyr Lys Gln Arg Val Lys Asn Lys  
Gly Arg Arg Ile Ala Tyr Leu [2].

4. An antibody in accordance with claim 1, in which said PACAP is PACAP27NH<sub>2</sub> consisting of an amino acid sequence represented by formula [3]:

His Ser Asp Gly Ile Phe Thr Asp Ser Tyr  
Ser Arg Tyr Arg Lys Gln Met Ala Val Lys  
Lys Tyr Leu Ala Ala Val Leu-NH<sub>2</sub> [3].

5. An antibody in accordance with claim 1, in which said antibody specifically binds to at least one peptide selected from the group consisting of PACAP27NH<sub>2</sub> represented by formula [3]; partial peptides of PACAP represented by formulae [4], [5], [6] and [7]; and vasoactive intestinal polypeptide represented by formula [8]:

PACAP (4-27)

Gly Ile Phe Thr Asp Ser Tyr  
Ser Arg Tyr Arg Lys Gln Met Ala Val Lys  
Lys Tyr Leu Ala Ala Val Leu [4]

PACAP (1-13)

His Ser Asp Gly Ile Phe Thr Asp Ser Tyr  
Ser Arg Tyr [5]

PACAP(14-38)NH<sub>2</sub>

Arg Lys Gln Met Ala Val Lys



- 71 -

Lys Tyr Leu Ala Ala Val Leu Gly Lys Arg

Tyr Lys Gln Arg Val Lys Asn Lys-NH<sub>2</sub> [6]PACAP(31-38)NH<sub>2</sub>Tyr Lys Gln Arg Val Lys Asn Lys NH<sub>2</sub> [7]

VIP (human)

1 5 10

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-

11 15 20

Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-

21 25 28

Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH<sub>2</sub> [8].

6. An antibody in accordance with claim 1, in which said antibody specifically binds to an N-terminal portion of said PACAP, said antibody reacting with PACAP38NH<sub>2</sub>, PACAP27NH<sub>2</sub>, PACAP(1-13) and PACAP(4-27), and not reacting with PACAP(14-38)NH<sub>2</sub> and PACAP(31-38)NH<sub>2</sub>.

7. An antibody in accordance with claim 1, in which said antibody specifically binds to a region from an N-terminal portion to a central portion of said PACAP, said antibody reacting with PACAP38NH<sub>2</sub>, PACAP27NH<sub>2</sub> and PACAP(4-27), and not reacting with PACAP(1-13), PACAP(14-38)NH<sub>2</sub> and PACAP(31-38)NH<sub>2</sub>.

8. An antibody in accordance with claim 1, in which said antibody specifically binds to a region from a C-terminal portion to a central portion of said PACAP, said antibody reacting with PACAP38NH<sub>2</sub> and PACAP(14-38)NH<sub>2</sub>, and not reacting with PACAP27NH<sub>2</sub>, PACAP(4-27), PACAP(1-13) and

- 72 -

PACAP(31-38)NH<sub>2</sub>.

9. An antibody in accordance with claim 1, in which said antibody specifically binds to a C-terminal portion of PACAP38, said antibody reacting with PACAP38NH<sub>2</sub>, PACAP(14-38)NH<sub>2</sub> and PACAP(31-38)NH<sub>2</sub>, and not reacting with PACAP27NH<sub>2</sub>, PACAP(4- 27), PACAP(1-13) and VIP.
10. An antibody in accordance with claim 6, in which said antibody shows a cross reactivity of 0.5% or less with VIP.
11. An antibody in accordance with claim 6, in which said antibody shows a cross reactivity of more than 0.5% with VIP.
12. An antibody in accordance with claim 7, in which said antibody shows a cross reactivity of 0.5% or less with VIP.
13. An antibody in accordance with claim 7, in which said antibody shows a cross reactivity of more than 0.5% with VIP.
14. Antibody in accordance with claim 4, in which said antibody specifically binds to a C-terminal peptide of PACAP27 or PACAP (11-27) represented by formula [9]:
- Ser Arg Tyr Arg Lys Gln Met Ala Val Lys

Lys Tyr Leu Ala Ala Val Leu-NH<sub>2</sub> [9].
15. A monoclonal antibody in accordance with claim 10, in which said monoclonal antibody is an antibody represented by PA-1Na.
16. A monoclonal antibody in accordance with claim 10, in which said monoclonal antibody is an antibody represented by PA-5Na.

17. A monoclonal antibody in accordance with claim 11, in which said monoclonal antibody is an antibody represented by PA-3Na.
18. A monoclonal antibody in accordance with claim 12, in which said monoclonal antibody is an antibody represented by PA-6Na.
19. A monoclonal antibody in accordance with claim 8, in which said monoclonal antibody is an antibody represented by PA-2Ca.
20. A monoclonal antibody in accordance with claim 9, in which said monoclonal antibody is an antibody represented by PA-1Ca.
21. A hybridoma cell which produces a monoclonal antibody specifically binding to PACAP or a precursor thereof.
22. A hybridoma cell which produces a monoclonal antibody claimed in claim 15.
23. A hybridoma cell which produces a monoclonal antibody claimed in claim 16.
24. A hybridoma cell which produces a monoclonal antibody claimed in claim 17.
25. A hybridoma cell which produces a monoclonal antibody claimed in claim 18.
26. A hybridoma cell which produces a monoclonal antibody claimed in claim 19.
27. A hybridoma cell which produces a monoclonal antibody claimed in claim 20.

28. A method for assaying PACAP, a partial peptide thereof or a precursor thereof in a test solution which comprises competitively reacting an antibody to the PACAP or the precursor thereof with the test solution and labeled PACAP, and then measuring a ratio of the labeled PACAP bound to said antibody.
29. A method for assaying PACAP, a partial peptide thereof or a precursor thereof in a test solution which comprises contacting the test solution with an antibody to the PACAP or the precursor thereof insolubilized on a carrier, then contacting therewith a labeled antibody to the PACAP or the precursor thereof, and measuring activity of a labeling agent on the insolubilized carrier.
30. A method in accordance with claim 29, in which one of the antibody to the PACAP or the precursor thereof insolubilized on the carrier and the labeled antibody to the PACAP or the precursor thereof is a polyclonal antibody, a monoclonal antibody or a complex thereof belonging to the antibody claimed in claim 6, the antibody claimed in claim 7 or the antibody claimed in claim 8, and the other antibody is a polyclonal antibody or a monoclonal antibody belonging to the antibody claimed in claim 9.
31. A method in accordance with claim 29, in which one of the antibody to the PACAP or the precursor thereof insolubilized on the carrier and the labeled antibody to the PACAP or the precursor thereof is a polyclonal antibody, a monoclonal antibody or a complex thereof belonging to the

antibody claimed in claim 6 or the antibody claimed in claim 7, and the other antibody is a polyclonal antibody or a monoclonal antibody belonging to the antibody claimed in claim 8.

32. A method in accordance with claim 29, in which one of the antibody to the PACAP or the precursor thereof insolubilized on the carrier and the labeled antibody to the PACAP or the precursor thereof is a polyclonal antibody or a monoclonal antibody belonging to the antibody claimed in claim 6, and the other antibody is a polyclonal antibody or a monoclonal antibody belonging to the antibody claimed in claim 7.

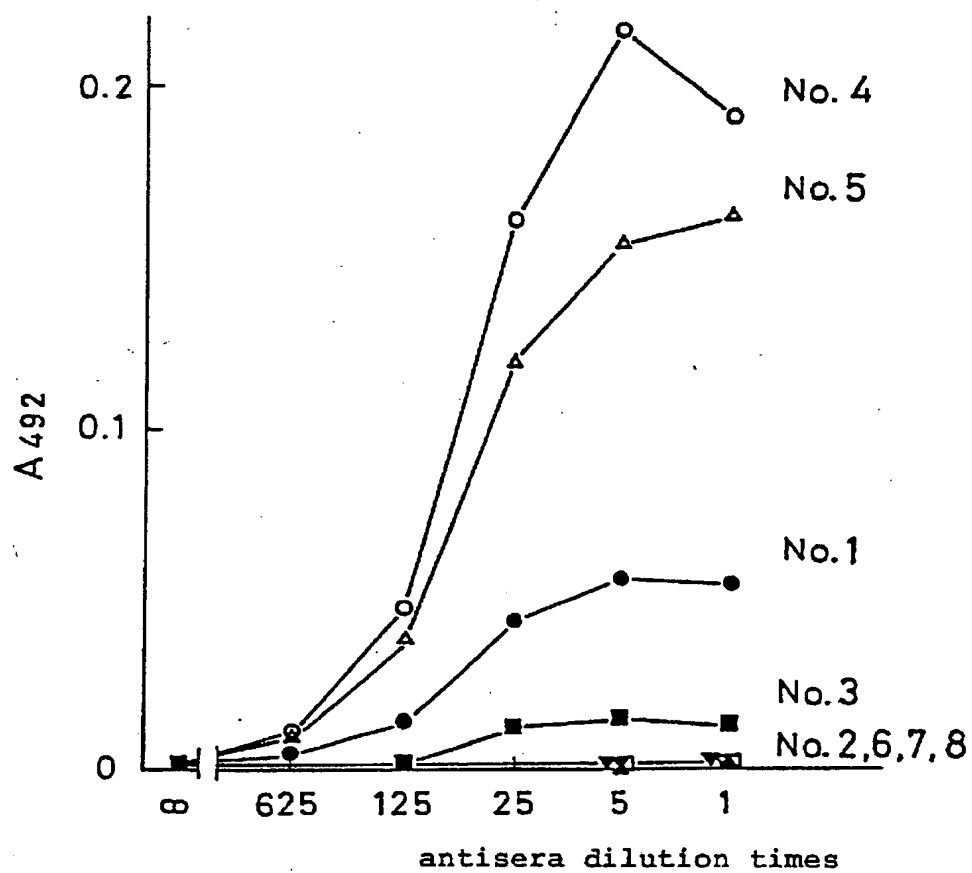
33. A method in accordance with claim 29, in which one of the antibody to the PACAP or the precursor thereof insolubilized on the carrier and the labeled antibody to the PACAP or the precursor thereof is a polyclonal antibody or a monoclonal antibody belonging to the antibody claimed in claim 6 or the antibody claimed in claim 7, and the other antibody is a polyclonal antibody or a monoclonal antibody belonging to the antibody claimed in claim 14.

34. A method in accordance with claim 30, in which one of the antibody to the PACAP or the precursor thereof insolubilized on the carrier and the labeled antibody to the PACAP or the precursor thereof is an antibody selected from the group consisting of PA-1Na, PA-3Na, PA-5Na, PA-6Na and PA-2Ca, or a complex thereof, and the other antibody is PA-1Ca.

35. A method in accordance with claim 31, in which one of the antibody to the PACAP or the precursor thereof insolubilized on the carrier and the labeled antibody to the PACAP or the precursor thereof is an antibody selected from the group consisting of PA-1Na, PA-3Na, PA-5Na and PA-6Na, or a complex thereof, and the other antibody is PA-2Ca.
36. A method in accordance with claim 32, in which one of the antibody to the PACAP or the precursor thereof insolubilized on the carrier and the labeled antibody to the PACAP or the precursor thereof is PA-5Na, and the other antibody is PA-6Na.
37. A method in accordance with claim 33, in which one of the antibody to the PACAP or the precursor thereof insolubilized on the carrier and the labeled antibody to the PACAP or the precursor thereof is an antibody selected from the group consisting of PA-1Na, PA-3Na, PA-5Na and PA-6Na, or a complex thereof, and the other antibody is the polyclonal antibody or the monoclonal antibody belonging to the antibody claimed in claim 14.
38. A method in accordance with claim 35, in which the antibody to the PACAP or the precursor thereof insolubilized on the carrier is PA-2Ca and the labeled antibody to the PACAP or the precursor thereof is PA-6Na.

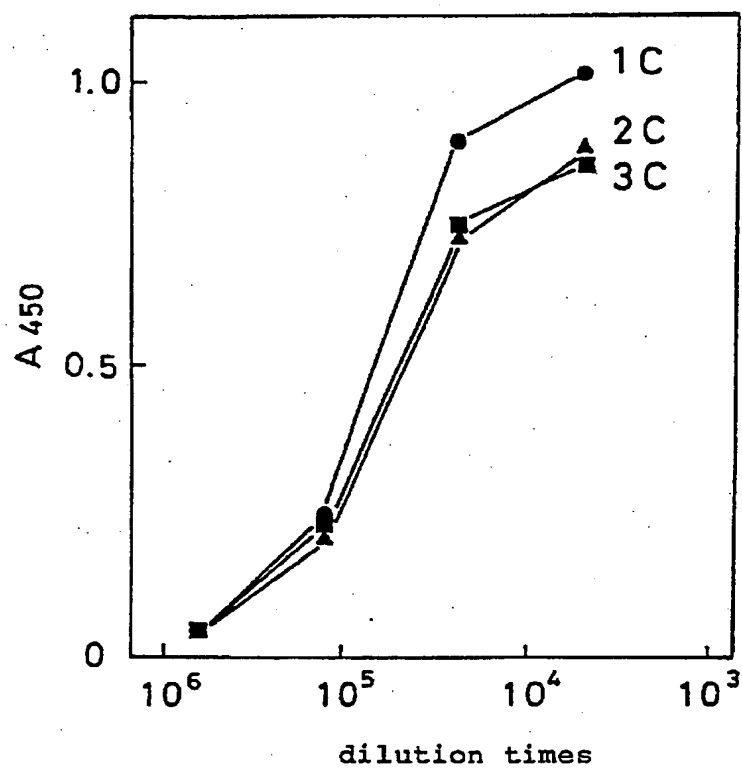
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Fig. 1



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Fig. 2

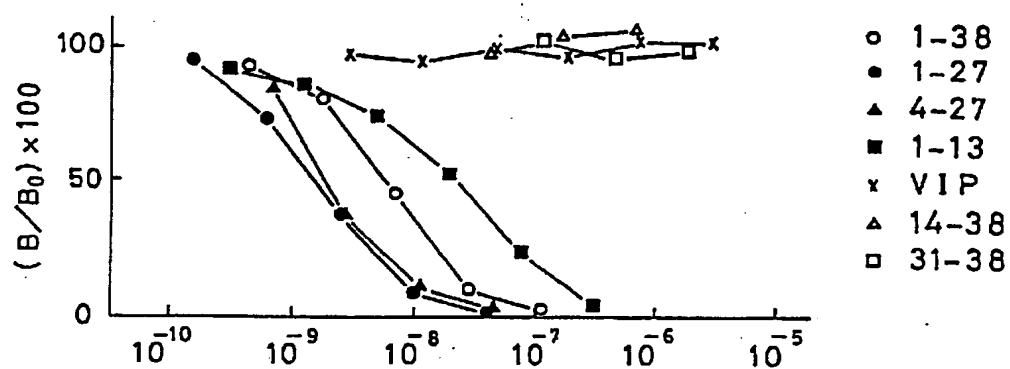




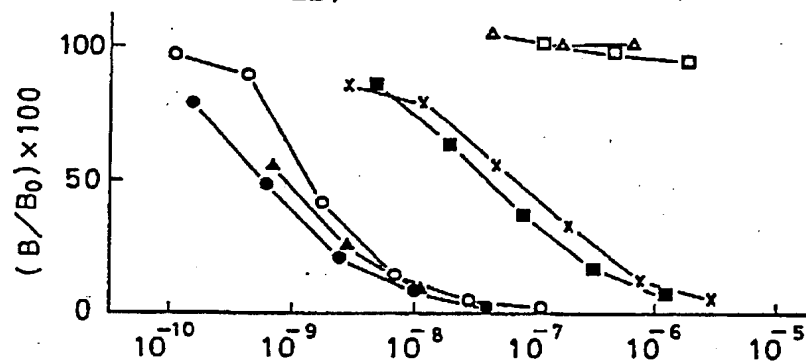
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Fig. 3-1

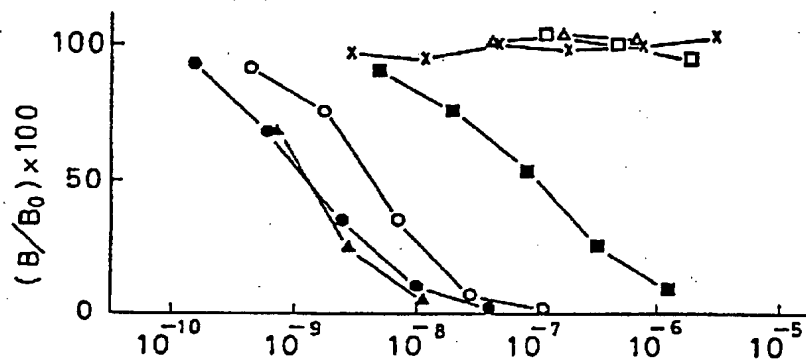
(a) PA-1Na(class Ia)



(b) PA-3Na(class Ib)



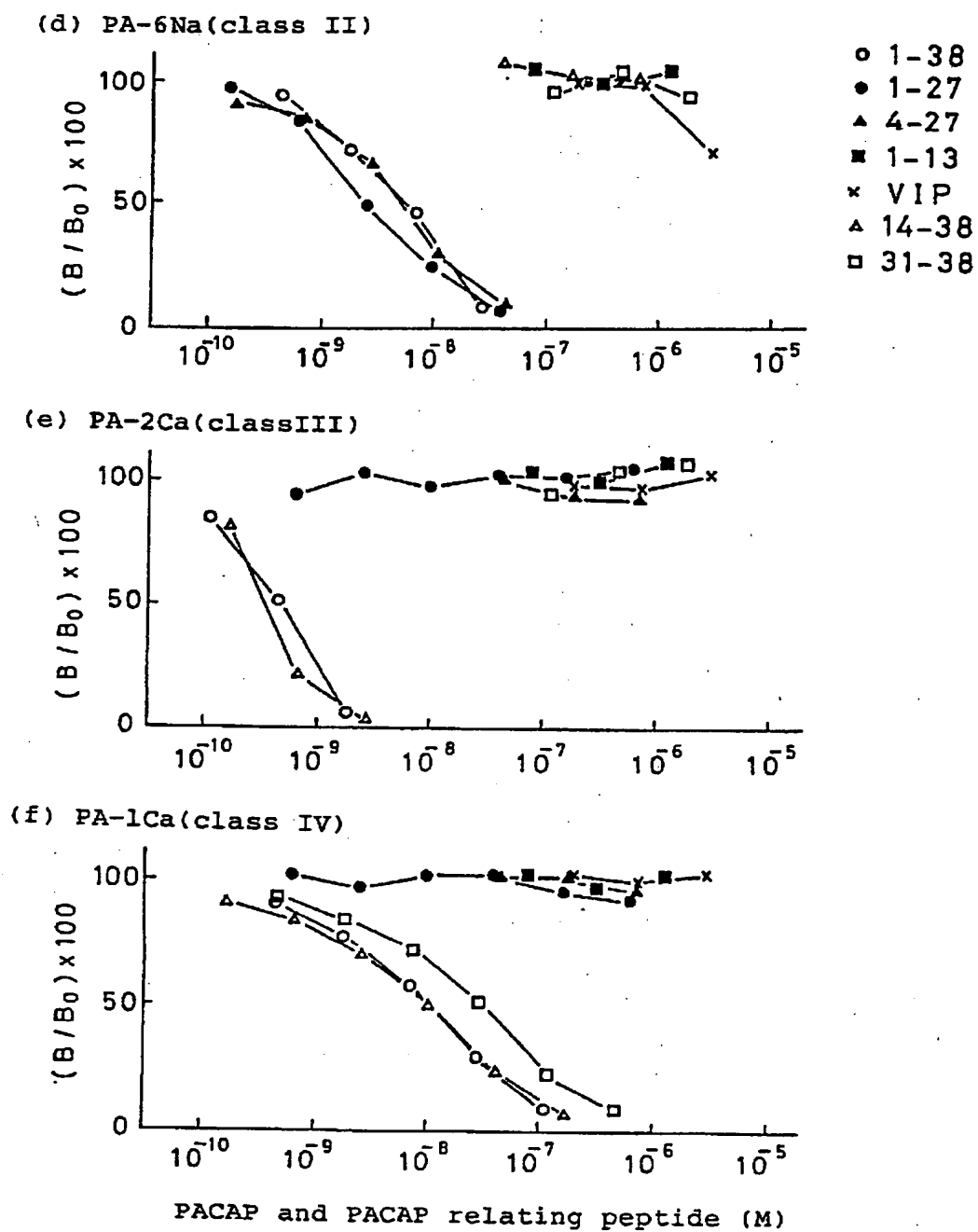
(c) PA-5Na(class Ia)



PACAP and PACAP relating peptide (M)

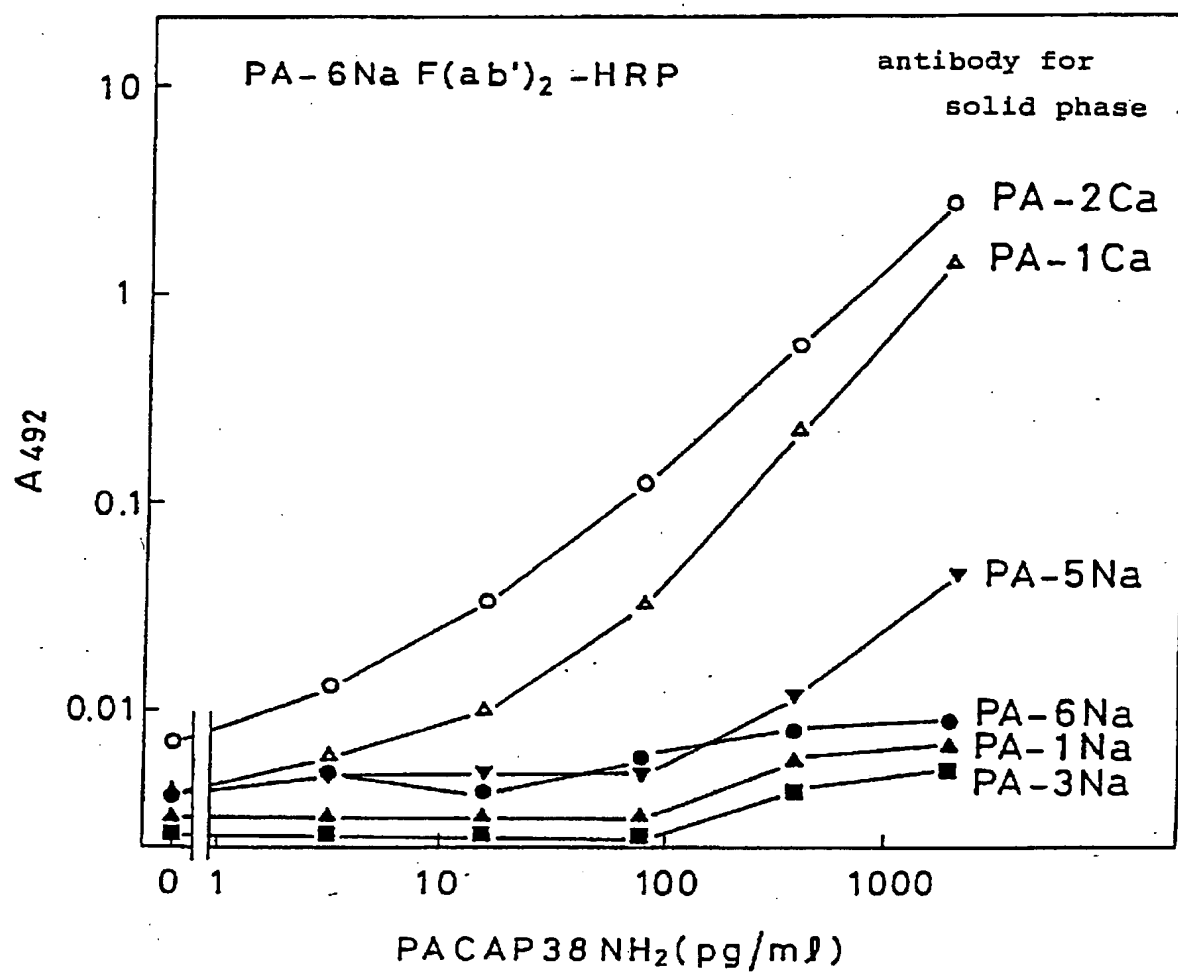
4/14

Fig. 3-2



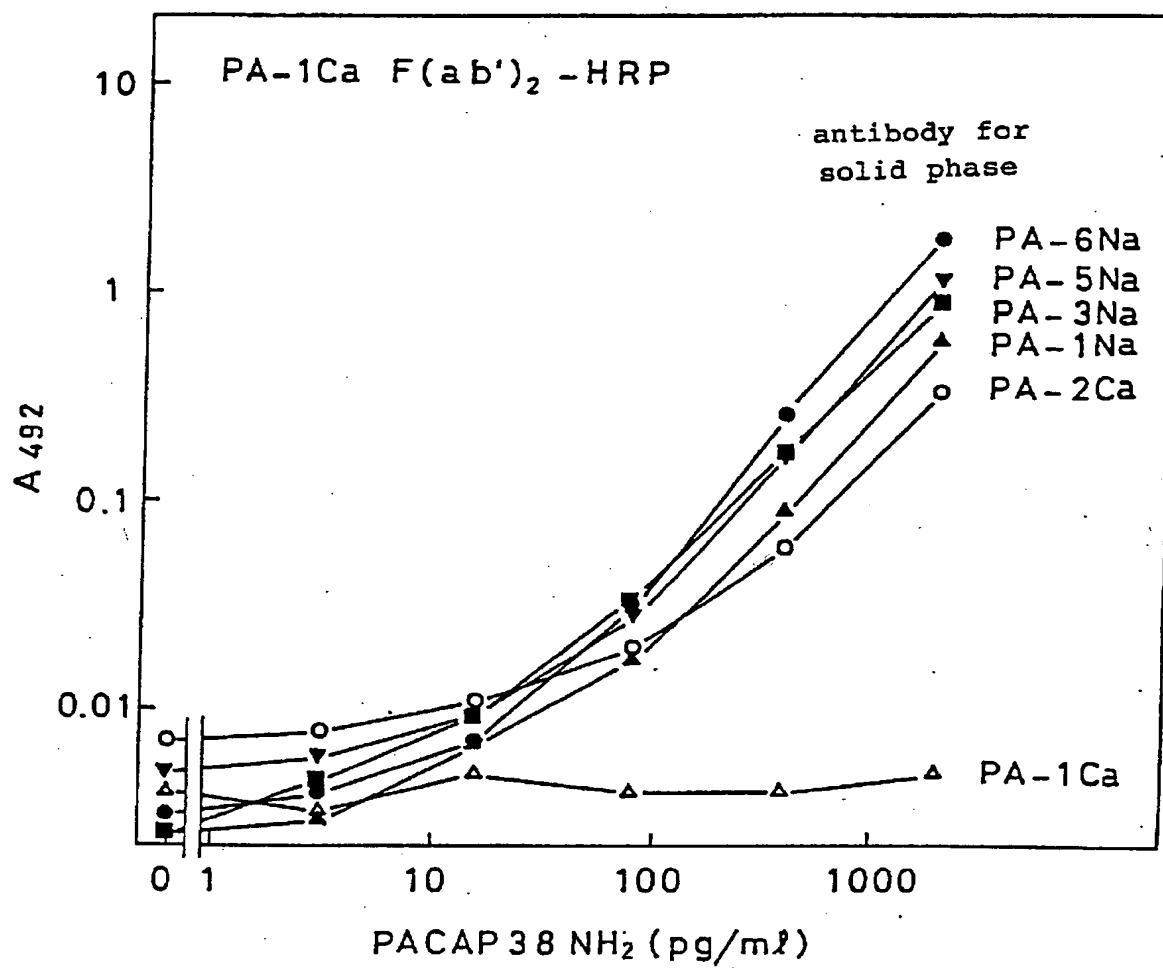
5/14

Fig. 4



6/14

Fig. 5



7/14

Fig. 6

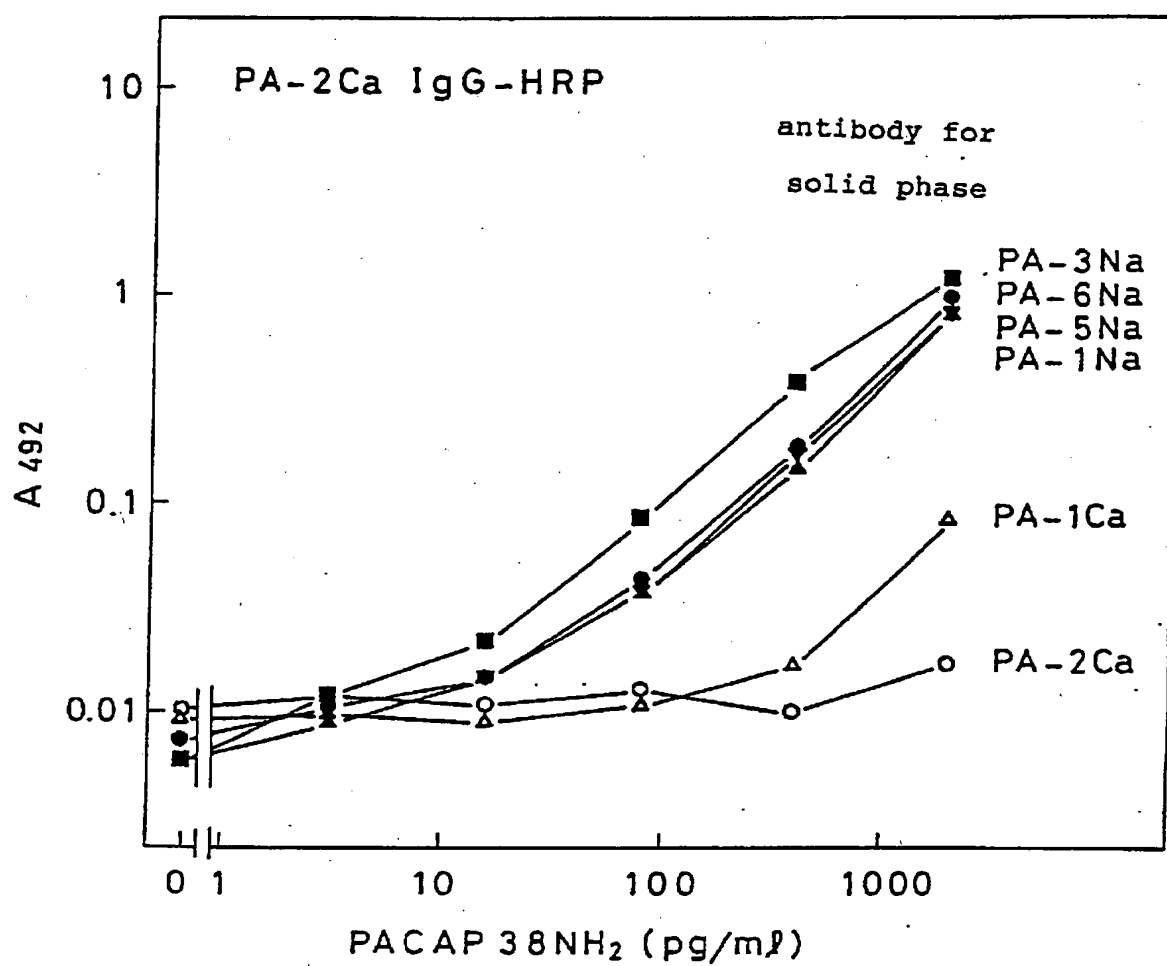
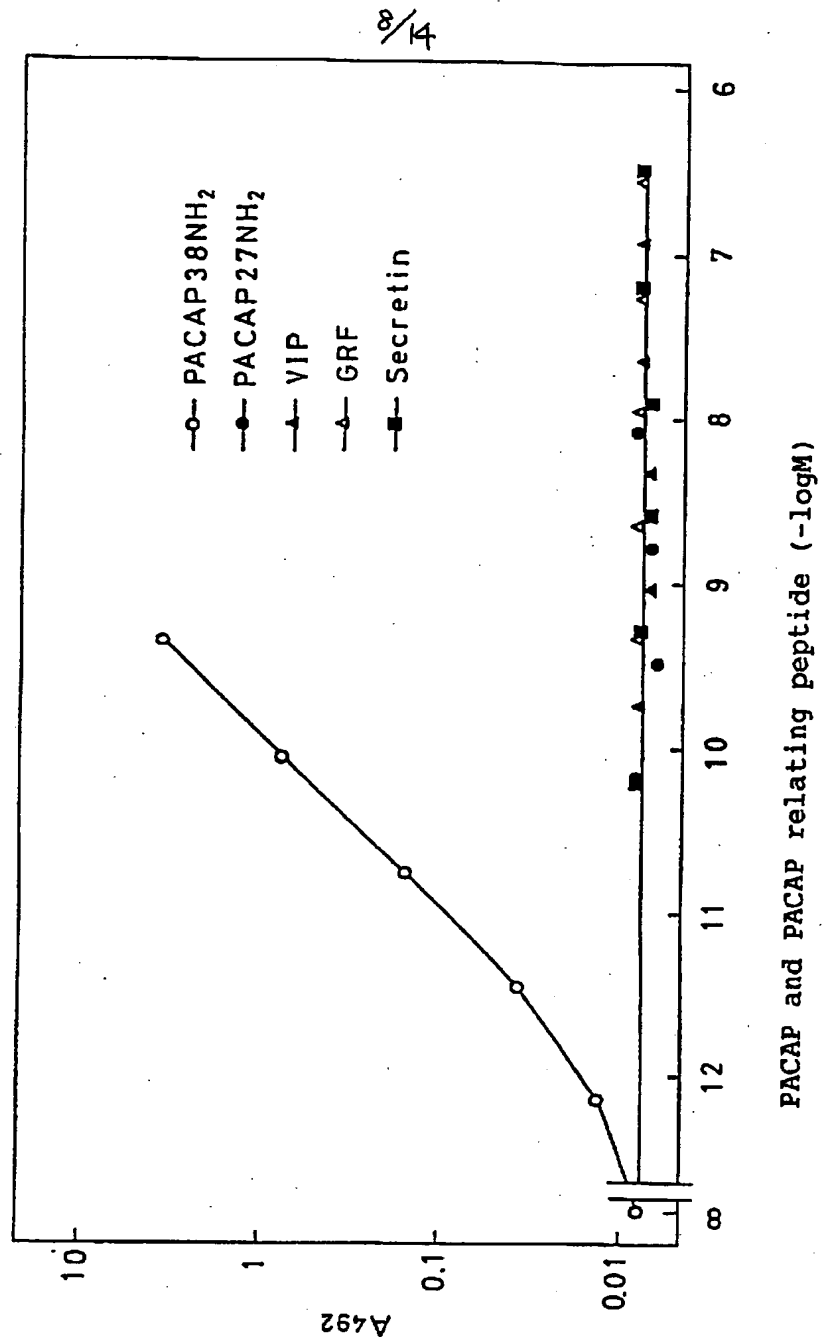
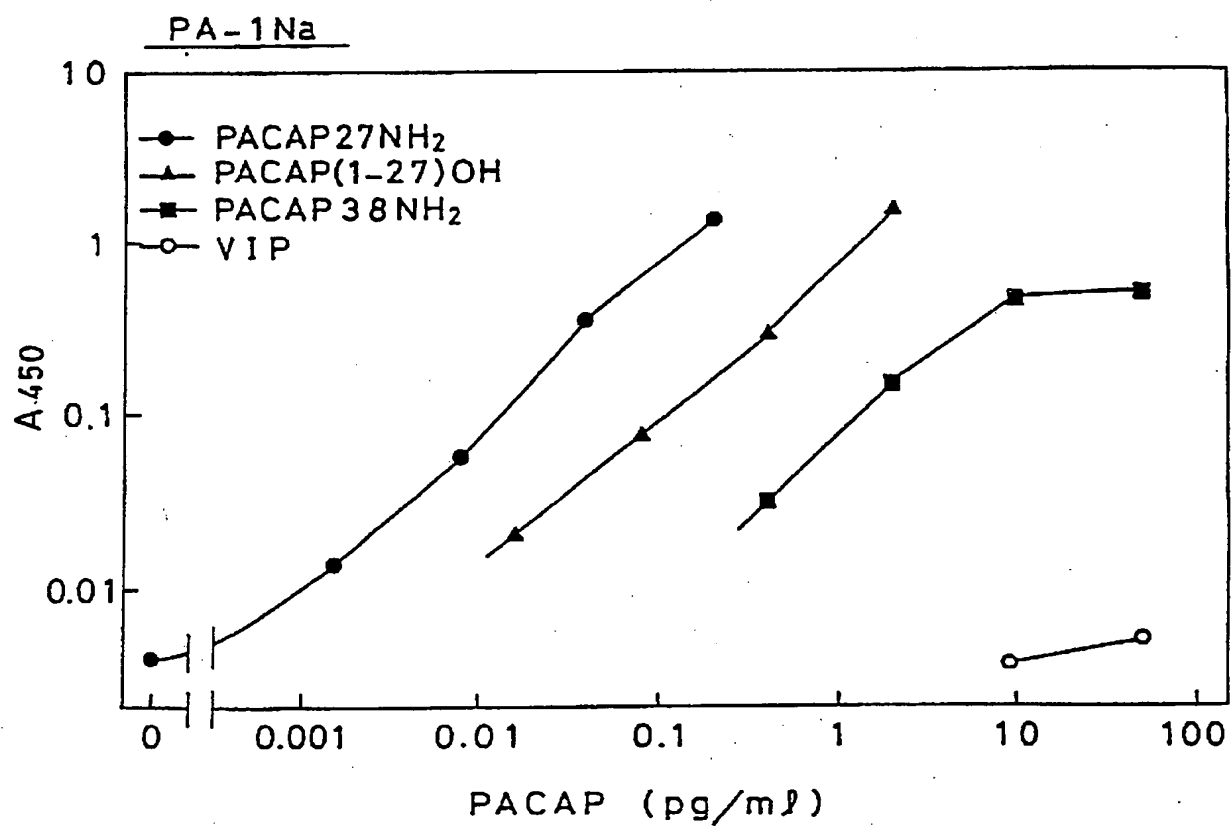


Fig. 7



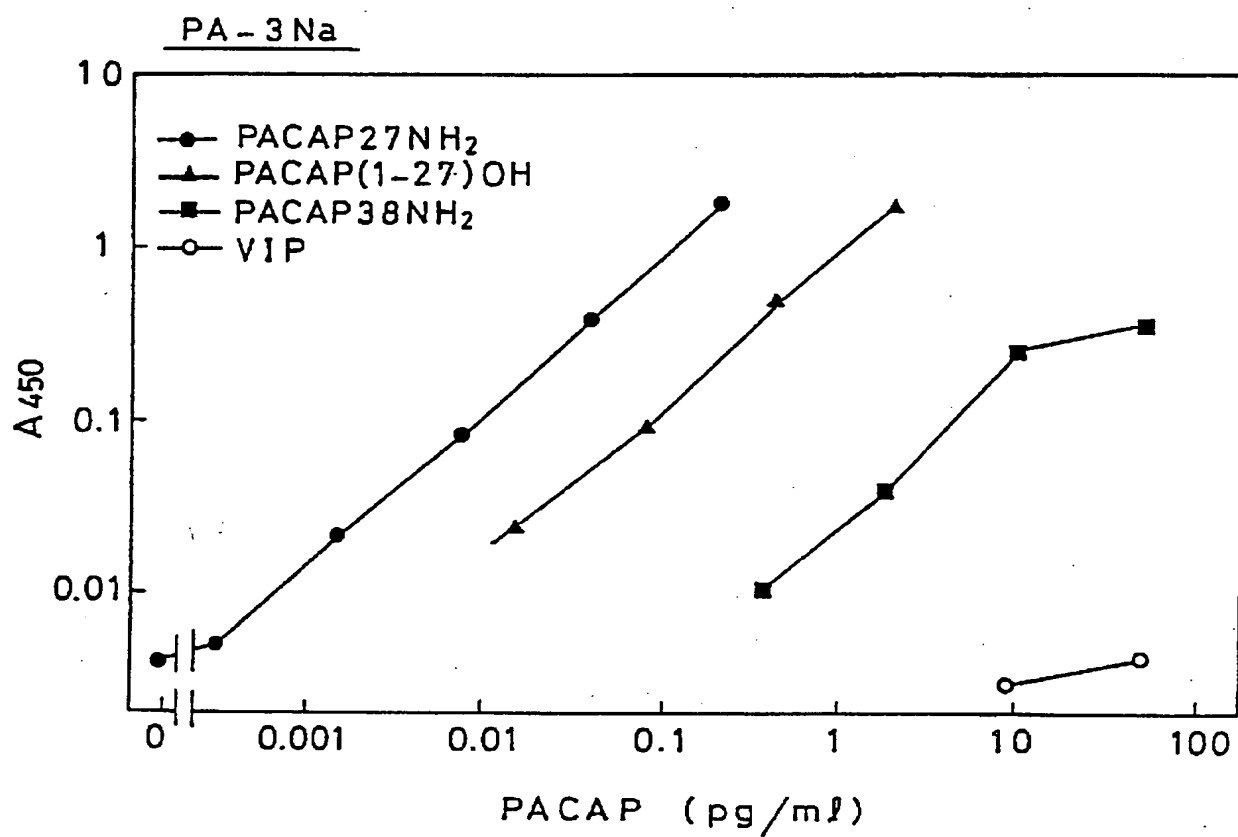
9/14

Fig. 8



10/14

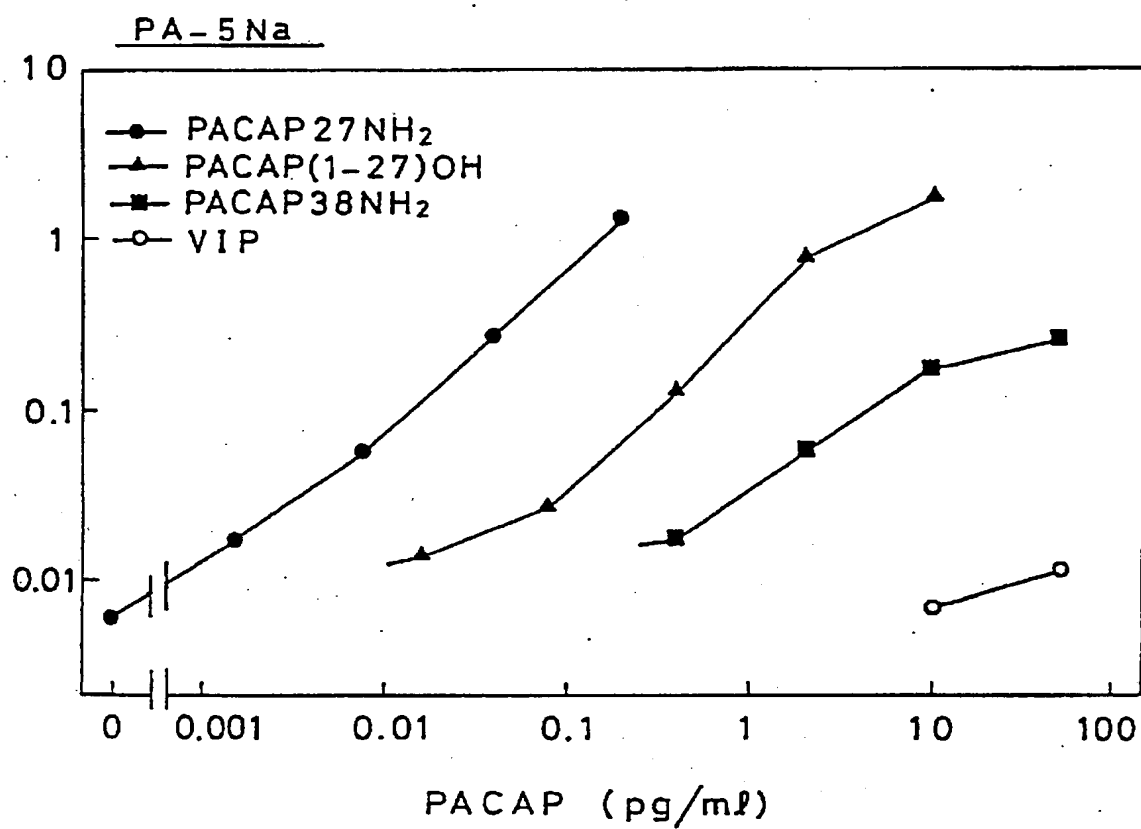
Fig. 9





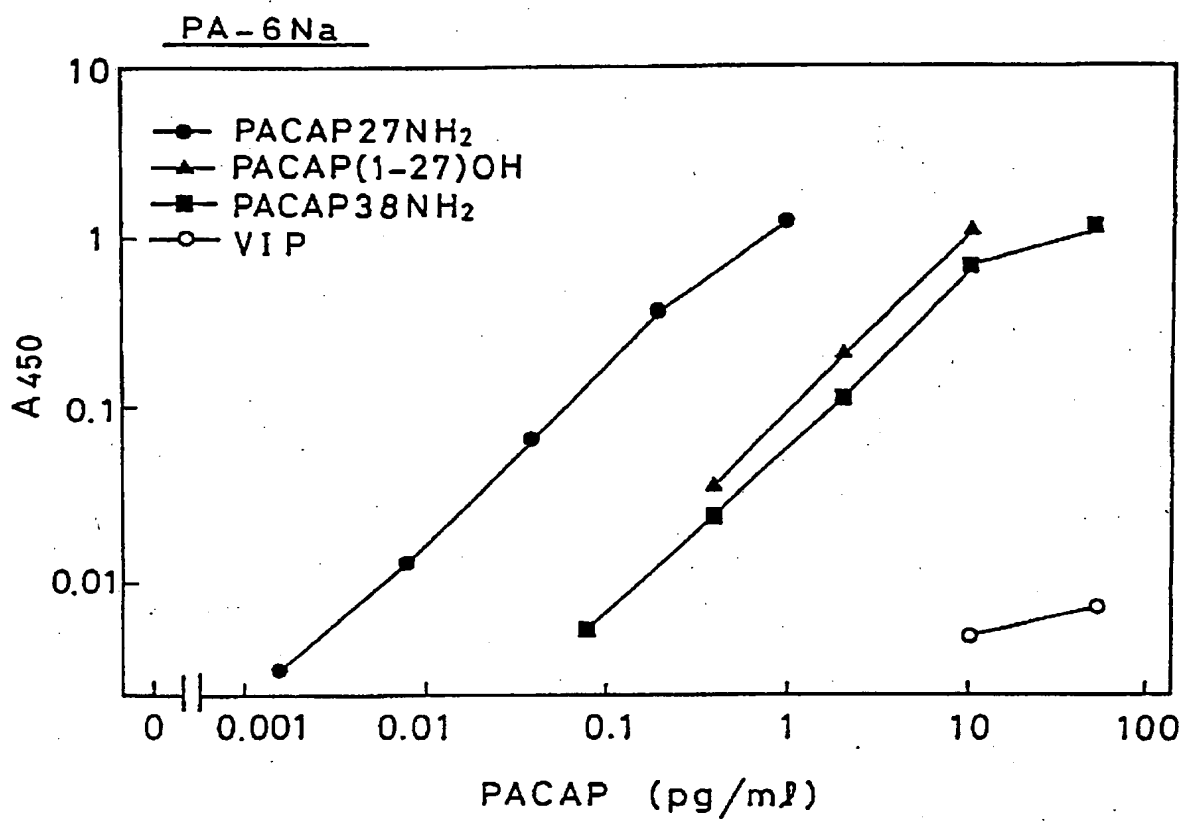
11/14

Fig. 10



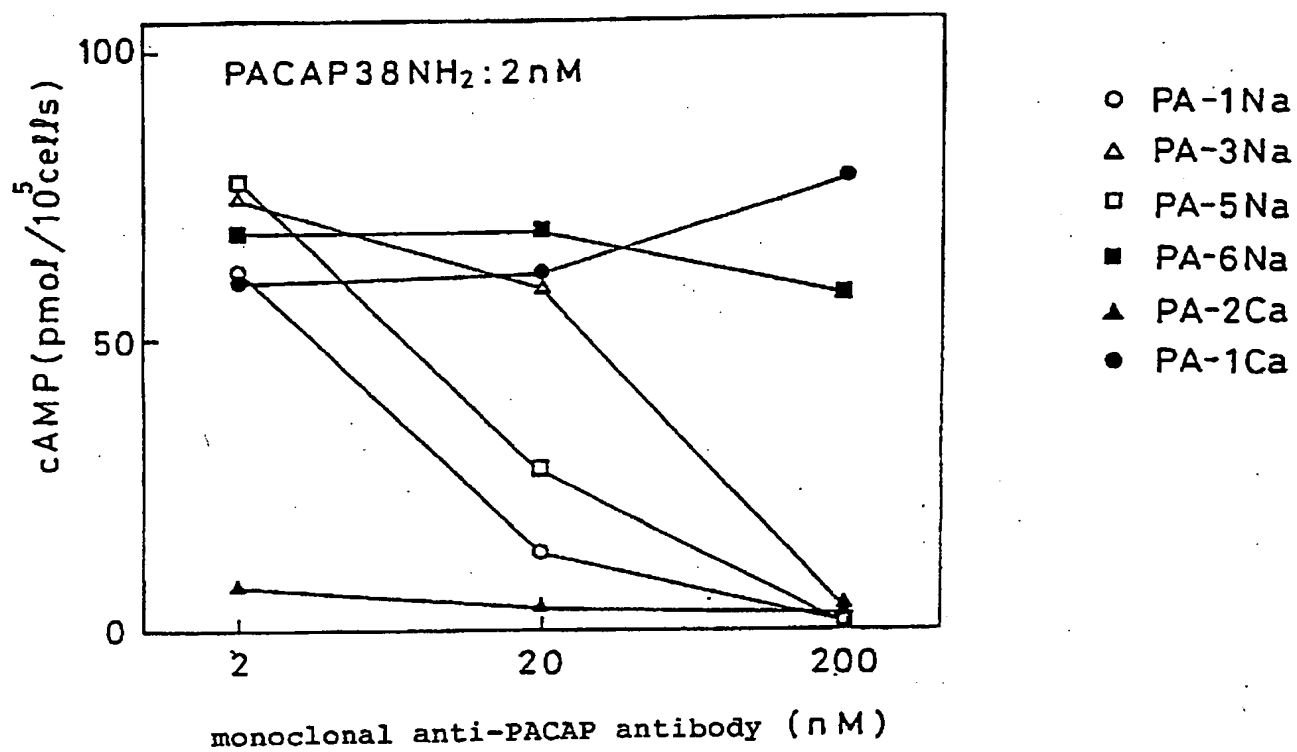
12/14

Fig. 11



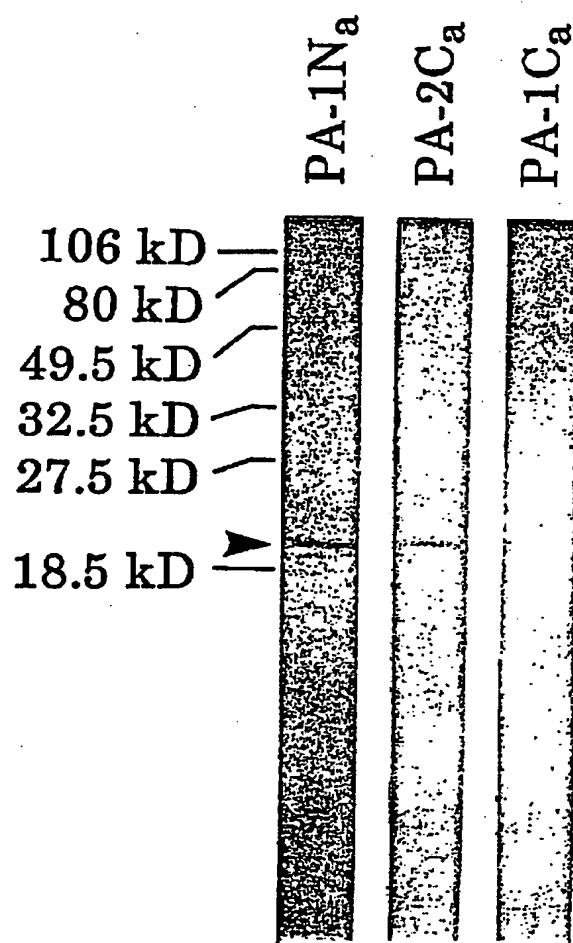
13/14

Fig. 12



$^{14}$ /<sub>14</sub>

Fig. 13



## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 25, line 8 of the description <sup>1</sup>A. IDENTIFICATION OF DEPOSIT<sup>2</sup>Further deposits are identified on an additional sheet ☐<sup>3</sup>Name of depositary Institution<sup>4</sup>

IFO: Institute for Fermentation, Osaka

FRI: Fermentation Research Institute, Agency of Industrial Science and Technology Ministry of International Trade and Industry

Address of depositary Institution (including postal code and country)<sup>4</sup>

IFO: 17-85, Juso-honmachi 2-chome, Yodogawa-ku, Osaka-shi, Osaka 532 Japan

FRI: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki 305 Japan

Date of deposit <sup>5</sup>

IFO: 27. 02. 90

FRI: 16. 03. 91

Accession Number <sup>6</sup>

IFO-50225

FERM BP-2811

B. ADDITIONAL INDICATIONS<sup>7</sup> (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE <sup>3</sup> (if the indications are not for all designated States)

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D. SEPARATE FURNISHING OF INDICATIONS<sup>8</sup> (leave blank if not applicable)


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27 May, 1991 (27.05.91)

T. Shimomichi   
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## MICROORGANISMS

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Date of deposit <sup>5</sup>

IFO: 27. 02. 90

FRI: 16. 03. 91

Accession Number <sup>6</sup>

IFO-59226

FERM BP- 2812

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D. SEPARATE FURNISHING OF INDICATIONS<sup>8</sup> (leave blank if not applicable)

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## MICROORGANISMS

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FRI: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki 305 Japan

Date of deposit <sup>5</sup>

IFO: 27. 02. 90

FRI: 16. 03. 91

Accession Number <sup>6</sup>

IFO- 50227

FERM BP- 2813

B. ADDITIONAL INDICATIONS<sup>7</sup> (leave blank if not applicable). This information is continued on a separate attached sheet ☐

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## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 25, line 11 of the description <sup>1</sup>A. IDENTIFICATION OF DEPOSIT<sup>2</sup>Further deposits are identified on an additional sheet ☐ <sup>3</sup>Name of depositary Institution<sup>4</sup>

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Date of deposit <sup>5</sup>

IFO: 27. 02. 90

FRI: 16. 03. 91

Accession Number <sup>6</sup>

IFO- 50228

FERM BP- 2814

B. ADDITIONAL INDICATIONS<sup>7</sup> (leave blank if not applicable). This information is continued on a separate attached sheet ☐

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## MICROORGANISMS

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Date of deposit <sup>5</sup>

IFO: 27. 02. 90

FRI: 16. 03. 91

Accession Number <sup>6</sup>

IFO- 50229

FERM BP- 2815

B. ADDITIONAL INDICATIONS<sup>7</sup> (leave blank if not applicable). This information is continued on a separate attached sheet ☐

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## MICROORGANISMS

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Date of deposit <sup>5</sup>

IFO: 27. 02. 90

FRI: 16. 03. 91

Accession Number <sup>6</sup>

IFO- 50230

FERM BP-2816

B. ADDITIONAL INDICATIONS<sup>7</sup> (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

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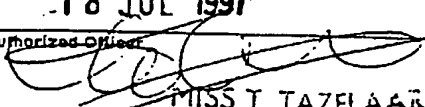
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27 May, 1991 (27.05.91)

T. Shimomichi   
(Authorized Officer)

# INTERNATIONAL SEARCH REPORT

International Application No PCT/JP 91/00354

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classifications apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC <b>IPC5: C 12 P 21/08, G 01 N 33/53, C 07 K 15/00, C 12 N 5/16</b>		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
IPC5	G 01 N; C 07 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
P,X	EP, A2, 0404034 (TAKEDA CHEMICAL INDUSTRIES, LTD) 27 December 1990, see page 13, line 49 - line 51  --	1-3,5, 28,29
P,X	Dialog Information Service, File 154: Medline, accession no. 07391864, Koves K et al: "Antisera against synthetic PACAP27 immunosorbent specific to PACAP27 and PACAP38", & Endocrinology Jul 1990, 127 (1) p264-71  --	1-5,14, 28,29
X	Chemical Abstracts, volume 99, no. 15, 10 October 1983, (Columbus, Ohio, US), Gozes, Iilana et al.: "Monoclonal antibodies against vasoactive intestinal polypeptide: studies of structure and related antigens", see page 128, abstract 116561g, & J. Neurochem. 1983, 41( 2), 549- 555  --	1,5,6, 11,13
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
4th June 1991	18 JUL 1991	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	 MISS T. TAZELAAR	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Chemical Abstracts, volume 86, no. 25, 20 June 1977, (Columbus, Ohio, US), Yanaihara, Noboru et al.: "Immunological aspects of secretin, substance P, and VIP", see page 412, abstract 187443, & Gastroenterology 1977, 72( 4), 803-810  --	1,5
X	Recueil, Journal of the Royal Netherlands Chemical Society, vol. 101, No. 11, November 1982, D. Voskamp et al.: "The role of histidine in the gastrointestinal hormone secretin ", see page 393 - page 396 fig 1 and page 394 right column, 4th paragraph  --	1,5
X	British medical bulletin, vol. 30, No. 1, 1974, J.S. Woodhead et al.: "The Immunoradiometric assay and related techniques ", see page 44, column - page 49 page 47, right column, last paragraph-page 48 left column  --	1-5
A	Jens F. Rehfeld "Sequence-specific radioimmunoassays for regulatory peptides", 1984, Elsevier Science Publishers B.V., see page 129-page 138  -- -----	1,5, 28

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SA 45601

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0404034	27/12/90	NONE	

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